Vera F. Dolan
454 Beltrami Drive, Ukiah, California 95482-8745
707.463.3200 / dolanvp@consultancy.com

EDUCATION and PROFESSIONAL CERTIFICATIONS

MSPH University of North Carolina at Chapel Hill, Occupational Epidemiology, 1981
BA The Johns Hopkins University, Public Health, 1979
FALU Fellow of the Academy of Life Underwriting, 1998
ELS Editor in the Life Sciences, 2003

EXPERIENCE

Principal, VFD Consulting, Inc., 1989 to Present
Providing life and health insurance and related industries mortality and underwriting research, market research, product development, business development and technology, underwriting education and training, and litigation support to multinational clients, including:

AIG American General Life Cos.
American Family Life Insurance Co.
AVS Underwriting
Bank of America
Calypte Biomedical
Canada Life Reinsurance
Charles Schwab & Co.
Clinical Reference Laboratory
decydeWARE
Edwards Lifesciences
EMSI (formerly PMSI)
Farmers Mutual Insurance Co.
FaxWatch
Federal Home Loan Bank (San Francisco)
First Financial Underwriting Services
General American Life Insurance Co.
Golden Gateway Financial
Hank George, Inc.
Health Net of California
Heritage Labs
HSBC Insurance Services
Independent Order of Foresters
Insure.com
Lab*One*
Life Settlement Consulting & Management
Los Angeles County Crime Laboratories
MetLife Canada
MIB Group
Millennium Pharmaceuticals
Mutual of Omaha
North Coast Opportunities
On The Risk – The Journal of the Academy of Life Underwriting
PartnerRe Life (formerly Winterthur Life Reinsurance)
Pacific Blue Cross
PPO Oklahoma
Protective Life Insurance Co.
Safe Harbor Resources
Sagicor Financial Corporation
Society of Actuaries
Thomson Management Solutions
21st Services
Wells Fargo Bank
Western Reserve Life Assurance Co. of Ohio
Xcelerate Corporation

GOVERNMENT EXHIBIT 12 1:14cv119

Vera F. Dolan
Curriculum Vitae, continued

Expert witness services provided to the following:

Multiple retentions:

Barger & Wolen, LLP, New York, NY and Irvine, CA
Lewis & Roca, Phoenix, AZ
U.S. Department of Justice

Multiple retentions, expert witness brokers:

Consolidated Consultants
ForensisGroup
Thomson Reuters Expert Witness Service

Single cases:

Andres, Andres & Moore, Santa Ana, CA
Andrews Kurth, Dallas, TX
Campano Law Group, Lancaster, CA
Carlin & Buchsbaum, Long Beach, CA
City of Torrance, Torrance, CA
Corr Cronin Michelson Baumgardner Preece, Seattle, WA
Cozen O'Connor, Wilmington, DE
Dressler Law Office, New Haven, CT
Donahue Horrow, El Segundo, CA
Epstein Becker & Green, Los Angeles, CA
Evan Freirich, PC, Boulder, CO
Fallgatter Farmand & Catlin, Jacksonville, FL
Friedman, Rubin & White, Anchorage, AL
Gilchrist & Rutter, Santa Monica, CA
Graves & King, LLP, Riverside, CA
Greenberg Traurig, LLP, Denver, CO
Jardine Law Office, DeForest, WI
Johnson Schachter & Lewis, Sacramento, CA
Katten Muchin Rosenman LLP, Los Angeles, CA
Koch, Degn and Gomez LLP, Visalia, CA
Law Offices of Cary T. Tanaka, Honolulu, HI
Law Offices of Clyde I. Butts, Walnut Creek, CA
Law Office of Katherine MacKinnon, St. Louis Park, MN
Law Offices of Michael Jay Green, Honolulu, HI
Law Offices of Robert A. Brenner, Woodland Hills, CA
Littler Mendelson, PC, San Jose, CA
MacMorris & Carbone, Stockton, CA
McArdle, Frost & Brinton, Chicago, IL
McDowell & Osburn, Manchester, NH
McFall, Burnett & Brinton, Manteca, CA
Milavetz, Gallop & Milavetz, Edina, MN
Myers & Gomel, Las Vegas, NV
Renaud Cook Drury Mesaros PA, Phoenix, AZ
Saur Law Office, Laguna Hills, CA
Schumacher, Francis & Nelson, Charleston, WV
Segal and Kirby LLP, Sacramento, CA
Shook, Hardy & Bacon, Kansas City, MO
Sigelman Law Corporation, Beverly Hills, CA
Stanley Law Offices, Syracuse, NY
Stennett & Casino, San Diego, CA
The Healy Law Firm, Chicago, IL
Webb Bordson Law Group, San Diego, CA
Wilson, Elser, Moskowitz, Edelman & Dicker, San Diego, CA

Vera F. Dolan
Curriculum Vitae, continued

Consulting Projects – Underwriting

- Life Underwriting Requirements Research, 2004 to Present Providing methodologies, analysis and write-up of insurance testing laboratory data to initiate new products and services. Conducting cohort mortality studies to improve life underwriting risk assessment.
- Life Policy and Procedures Manual, 2014 Revised and updated life insurance policy and procedures manual based on current underwriting and business operations.
- Health Medical Underwriting Manual, 2010 Adapted medical underwriting manual for supplemental health insurance products.
- Structured Settlement Underwriting Manual, 2008 Developed underwriting manual for structured settlements based on current processes.
- Life Claims Audit, 2007 Performed random selection of cases and designed case data extraction sheet for large life insurance claims study.
- Critical Illness and Disability Income Training, 2006 Prepared and presented training to life underwriters in critical illness and disability income underwriting.
- Underwriting Manual Research and Writing, 2006 Adapted traditional life underwriting manual for simplified life, disability and waiver of premium products.
- Life Settlement Experience Analysis, 2006 Analysis of life settlement mortality to determine predictive factors.
- Life Underwriting Research, 2001 to 2003 Constructed life tables from medical literature to validate life underwriting guidelines. Produced quantitative and qualitative life underwriting manual guidance.
- Life Settlement Underwriting Research, 1998 to 2004 Provided model and design of automated life expectancy calculation system, data collection instruments, evaluation scoring and impairment risk factor weights.
- Insurance Services Product Development, 1997 to 1998 Provided analysis of disease mortality, incidence and prevalence to develop new insurance services product.
- Pension Underwriting Research, 1994 Provided biostatistical research basis to a multinational consortium for a new type of impaired risk European pension product.

Consulting Projects - Business Development and Technology

- Simplified Products Survey and Report, 2005 Conducted survey of top-selling simplified products for underwriting and business process lessons learned.
- New Business Process Review and Optimization, 2004 to 2005 Examined current life new business processes to identify opportunities for decreasing redundancies, handoffs, and inefficiencies.
- Underwriting Decision Software Development, 2004 to 2005 Product and market development of life underwriting software that uses advanced logic engine
- Freelance Writing and Editing, 2004 Editing and writing summaries of medical articles, medical conferences, and insurance topics for physicians and consumers.
- Underwriting Operations Annual Report, 2004 Assisted in editing, writing and analysis of metrics presented in life underwriting operations report for 2003.
- Business Process Improvement, 2003 Interviewed underwriting and business support staff to prepare future improvement in life insurance business process workflow.

Vera F. Dolan
Curriculum Vitae, continued

Consulting Projects - Banking

- Enterprise Technology Plan, 2000 Collaborated on design of three-year technology plan for a Federal bank.
- Financial Services Experience Analysis and Strategy, 1995 to 1998 Provided statistical analysis and models to select business and consumer customers for acquisition and retention programs. Estimated bank account life expectancies using actuarial life table methods.
- Human Resources Automation Plan, 1993 Collaborated in development of plan and prototype for automated human resources management.

Associate Editor, *On The Risk - Journal of the Academy of Life Underwriting*, 2004-Present
Contributing Editor, *On The Risk - Journal of the Academy of Life Underwriting*, 1994-1997

Kaiser Foundation Health Plan, Oakland, California
Medical Economics and Statistics, 1988 to 1989
Converted community-based pricing approach to experience-based rating for a $2.5 billion premium enterprise. Conducted underwriting market study of 20,000 employers. Conducted analyses to determine competitive pricing for 2+ million member optical and pharmaceutical programs.

Transamerica Occidental Life Insurance Company, Los Angeles, California
New Business Underwriting Research, 1985 to 1988
Provided analysis of company data and medical literature to support management and underwriting decisions. Developed original methods for actuarial analyses now used for automated systems underwriting $1.5 billion in annual business.

Lincoln National Reinsurance, Ft. Wayne, Indiana
Underwriting Research, 1982 to 1985
Provided analysis of company data and medical literature to support management and underwriting decisions.

University of North Carolina, Chapel Hill, North Carolina
Department of Biostatistics, 1981 to 1982
Performed statistical analyses for multinational clinical trials.

National Institute of Occupational Safety and Health, Cincinnati, Ohio
Industrywide Studies Branch, Biometry Section, Summer 1980
Performed proportionate mortality analysis of death certificate data for evidence of excess brain tumors among petrochemical workers. Performed analysis of medical records for evidence of excess birth anomalies among railroad workers exposed to dioxin.

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE

"Advantages of a Life Expectancy Using Life Insurance Underwriting and Life Settlement Methods in the Legal Setting" VFD Consulting, Inc., 2014

"Serum Globulin Predicts All-Cause Mortality for Life Insurance Applicants" J. Insurance Medicine, Vol. 44, No. 2, 2014, with Michael Fulks and Robert L. Stout

"Advantages of a Life Expectancy Using Life Insurance Underwriting and Life Settlement Methods in the Legal Setting" Defense Research Institute Life, Health and Disability Newsletter, Vol. 25, Issue 2, 2014.

"NT-proBNP as a Predictor of All-Cause Mortality in a Population of Insurance Applicants." On The Risk - Journal of the Academy of Life Underwriting, Vol. 30, No. 2, 2014, with Michael Clark, Valerie Kaufman, Michael Fulks and Robert L. Stout

"Beware That Low Urine Creatinine!" On The Risk - Journal of the Academy of Life Underwriting, Vol. 30, No. 1, 2014, with Michael Fulks and Robert L. Stout

"NT-proBNP as a Predictor of All-Cause Mortality in a Population of Insurance Applicants." J. Insurance Medicine, Vol. 44, No. 1, 2014, with Michael Clark, Valerie Kaufman, Michael Fulks and Robert L. Stout

"Scoring Life Insurance Applicants' Laboratory Results, Blood Pressure and Build to Predict All-Cause Mortality Risk" J. Insurance Medicine, Vol. 43, No. 3, 2012, with Michael Fulks and Robert L. Stout

"Urine Protein/Creatinine Ratio as a Risk Predictor in Non-Diabetics with Normal Renal Function" J. Insurance Medicine, Vol. 43, No. 2, 2012, with Michael Fulks and Robert L. Stout

"Trends in Mortality of Insurance Applicants with HIV Infection" J. Insurance Medicine, Vol. 43, No. 2, 2012, with Robert L. Stout and Michael Fulks

"PSA: What Values Predict Increased Mortality Risk?" On The Risk - Journal of the Academy of Life Underwriting, Vol. 27, No. 3, 2011, with Michael Fulks and Robert L. Stout

"Letter to the Editor: The Use of Modeling for Associating Test Values and Mortality Risk." J. Insurance Medicine, Vol. 43, No. 1, 2012, with Michael Fulks and Robert L. Stout

"Isolated Hematuria as a Mortality Risk Predictor" On The Risk - Journal of the Academy of Life Underwriting, Vol. 27, No. 4, 2011, with Michael Fulks and Robert L. Stout

"Highlights of the 2011 AHOU Annual Conference" On The Risk - Journal of the Academy of Life Underwriting, Vol. 27, No. 3, 2011

"Correction to article: Association of Cholesterol, LDL, HDL, Cholesterol/HDL and Triglyceride with All-Cause Mortality in Life Insurance Applicants." J. Insurance Medicine, Vol. 42, No. 2-4, 2011, with Michael Fulks and Robert L. Stout

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"CDT and Serum Alcohol: What Is the Risk?" On The Risk - Journal of the Academy of Life Underwriting, Vol. 27, No. 1, 2011, with Robert L. Stout and Michael Fulks

"Albumin and All-Cause Mortality Risk in Insurance Applicants" J. Insurance Medicine, Vol. 42, No. 1, 2010, with Michael Fulks and Robert L. Stout

"Mortality Associated with Positive Hepatitis C and B Test Results" On The Risk - Journal of the Academy of Life Underwriting, Vol. 26, No. 3, 2010, with Robert L. Stout and Michael Fulks

"Glucosuria as a Mortality Risk Predictor When Blood Is Not Collected" On The Risk - Journal of the Academy of Life Underwriting, Vol. 26, No. 2, 2010, with Robert L. Stout and Michael Fulks

"Mortality Associated with Positive Cocaine Test Results" On The Risk - Journal of the Academy of Life Underwriting, Vol. 26, No. 1, 2010, with Robert L. Stout and Michael Fulks

"Association of Cholesterol, LDL, HDL, Cholesterol/HDL and Triglyceride with All-Cause Mortality in Life Insurance Applicants" J. Insurance Medicine, Vol. 41, No. 4, 2009, with Michael Fulks and Robert L. Stout

"Letter to the Editor: The Authors Reply" J. Insurance Medicine, Vol. 41, No. 3, 2009, with Michael Fulks and Robert L. Stout

"Underwriting Implications of Elevated Carcinoembryonic Antigen" On The Risk - Journal of the Academy of Life Underwriting, Vol. 25, No. 3, 2009, with Robert L. Stout and Michael Fulks

"Mortality Associated with Bilirubin Levels in Insurance Applicants" J. Insurance Medicine, Vol. 41, No. 1, 2009, with Michael Fulks and Robert L. Stout

"Non-Cigarette Tobacco Use – What is the Risk?" On The Risk - Journal of the Academy of Life Underwriting, Vol. 25, No. 2, 2009, with Robert L. Stout and Michael Fulks

"Hemoglobin A1c and Mortality in Insurance Applicants: A 5-Year Follow-Up Study", On The Risk - Journal of the Academy of Life Underwriting, Vol. 25, No. 1, 2009, with Robert L. Stout and Michael Fulks

"Using Liver Enzymes as Screening Tests to Predict Mortality Risk" J. Insurance Medicine, Vol. 40, No. 4, 2008, with Michael Fulks and Robert L. Stout

"Underwriting Integrity: Lessons from the Subprime Mortgage Crisis" On The Risk - Journal of the Academy of Life Underwriting, Vol. 24, No. 4, 2008

"Highlights from the 2007 AHOU Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 24, No. 1, 2008

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"Increased Mortality Associated with Elevated Carcinoembryonic Antigen in Insurance Applicants" J. Insurance Medicine, Vol. 39, No. 4, 2007, with Robert L. Stout, Michael Fulks, Mark E. Magee and Luis Suarez

"Relationship of Hemoglobin A1c to Mortality in Nonsmoking Insurance Applicants" J. Insurance Medicine, Vol. 39, No. 3, 2007, with Robert L. Stout, Michael Fulks, Mark E. Magee and Luis Suarez

"Highlights from the 2006 AHOU Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 23, No. 1, 2007

"The Reproductive System" In: Intermediate Medical Life Insurance Underwriting ALU 201, 1st ed., Education Committee of the Academy of Life Underwriting, chapter 4, 2006

"Highlights from the 2006 CIU Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 22, No. 3, 2006

"Improvements in Cotinine Testing of Insurance Applicants" On The Risk - Journal of the Academy of Life Underwriting, Vol. 22, No. 2, 2006 with Robert L. Stout and Mark Magee

"Highlights from the LOMA Emerging Technology Conference: Understanding How to Use New Technology" On The Risk - Journal of the Academy of Life Underwriting, Vol. 22, No. 2, 2006

Panelist, "Contemplating Pandemic Risk" by Steven C. Siegel, The Actuary, Vol. 3, Issue 3, 2006

"Highlights of the 2005 AHOU Conference" On The Risk - Journal of the Academy of Life Underwriting, Vol. 22, No. 1, 2006

"Simplified Products: Something Old, Something New, and Something That May Just Make You Blue!" On The Risk - Journal of the Academy of Life Underwriting, with Ernest A. Testa, Vol. 22, No. 1, 2006

"2005 Simplified Product Survey: Final Report" for MIB Group, with Ernest A. Testa, January 2006

"Highlights of the 2005 CIU Conference" On The Risk - Journal of the Academy of Life Underwriting, Vol. 21, No. 4, 2005

"Underwriting for the New Millennium: Simplified Product Survey" NewsDirect, September 2005

"Sleep Problems and Accidents" On The Risk - Journal of the Academy of Life Underwriting, Vol. 21, No. 1, 2005

"Customer Relationship Management: A Reinsurer's Experience" On The Risk - Journal of the Academy of Life Underwriting, Vol. 20, No. 2, 2004

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"Current Concepts in the Insulin Resistance Syndrome" On The Risk - Journal of the Academy of Life Underwriting, with Robert Weir, Vol. 20, No. 1, 2004

"Highlights of the 2003 AAIM" On The Risk - Journal of the Academy of Life Underwriting, Vol. 20, No. 1, 2004

"Current Concepts in Multiple Sclerosis" On The Risk - Journal of the Academy of Life Underwriting, with Robert Weir, Vol. 19, No. 4, 2003

"Multiple Sclerosis" Canada Life Review Online, Third Quarter 2003, with Robert Weir

"Highlights of the 2003 CIU Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 19, No. 3, 2003

"Web-Assisted Inspection Reports: Part 2 of 2" On The Risk - Journal of the Academy of Life Underwriting, Vol. 19, No. 3, 2003

"Keynote Presentation to the Annual Meeting, Canadian Institute of Underwriters" Canada Life Review Online, Second Quarter 2003

"Web-Assisted Inspection Reports: Part 1 of 2" On The Risk - Journal of the Academy of Life Underwriting, On The Risk - Journal of the Academy of Life Underwriting, Vol. 19, No. 2, 2003

"Highlights of the 2002 AAIM" On The Risk - Journal of the Academy of Life Underwriting, Vol. 19, No. 1, 2003

Project Manager, "Report of the 2002 Automated Risk Assessment System Study" Thomson Management Solutions, February 2003

"Insulin Resistance Syndrome" Canada Life Review Online, Fourth Quarter 2002

"The New Paradigm of Coronary Artery Disease: Part 1. The Pathophysiology of Vulnerable Plaque" On The Risk - Journal of the Academy of Life Underwriting, with Robert Weir, Vol. 18, No. 4, 2002

"Orthostatic Hypotension" On The Risk - Journal of the Academy of Life Underwriting, with Robert Weir, Vol. 18, No. 3, 2002

"Highlights of the 2002 CLIMOA" On The Risk - Journal of the Academy of Life Underwriting, Vol. 18, No. 3, 2002

"Treatment of Localized Prostate Cancer" On The Risk - Journal of the Academy of Life Underwriting, Vol. 18, No. 2, 2002

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"Privacy Rules and Regulations: An Interview with Linda S. Kaiser, Esq." On The Risk - Journal of the Academy of Life Underwriting, Vol. 18, No. 1, 2002

"Treated Cholesterol; A Preferred Risk?" Canada Life Review Online, First Quarter 2002, with Robert Weir

"Body Mass Index and Mortality" On The Risk - Journal of the Academy of Life Underwriting, Vol. 17, No. 4, 2001, with Robert Weir

"Innovations in Underwriting Build" Canada Life Review No. 2, 2001

"Honoring Past HOLUA Presidents" On The Risk - Journal of the Academy of Life Underwriting, Vol. 17, No. 3, 2001

"Highlights of the 2001 HOLUA: An Underwriter's Odyssey" On The Risk - Journal of the Academy of Life Underwriting, Vol. 17, No. 3, 2001

"Genetic Testing Provides Challenge to Insurers" American Clinical Laboratory, Vol. 20, No. 4, 2001 pp. 18-9

"Highlights of the Spring 2001 Risk Appraisal Forum" On The Risk - Journal of the Academy of Life Underwriting, Vol. 17, No. 2, 2001

"Highlights of the 2000 IHOU: Gateway to Change" On The Risk- Journal of the Academy of Life Underwriting, Vol. 17, No. 1, 2001

"Progress in Establishing the Association of Home Office Underwriters" On The Risk - Journal of the Academy of Life Underwriting, Vol. 17, No. 1, 2001

"Innovative Technologies for Detecting Alcohol Abuse and Monitoring Drinking Status" American Clinical Laboratory, Vol. 20, No. 1, 2001

"Highlights of the 2000 CIU: Take a Risk!" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 4, 2000

"Alternative/Complementary Medicine" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 4, 2000

"Life Settlements" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 4, 2000, with Chris Cook

"Underwriting Settlements" In: Viatical and Life Settlements: The Challenge Facing the Life Insurance Industry, Jean Gora, ed., Life Office Management Association, Chapter 4, 2000

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"Detecting Alcohol Abuse and Monitoring Drinking Status" Lab*One* Insight, Fall 2000

"Building Corporate E-Commerce" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 3, 2000

"Highlights of the 2000 HOLUA: Brave New World" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 3, 2000

"Progress Report on the Formation of the New Underwriting Organization" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 3, 2000

"Highlights of the Spring 2000 Risk Appraisal Forum" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 2, 2000

"Les Grubin Hosts Risk Appraisal Forum in the City by the Bay" Broker World, Vol. 20, No. 6, 2000

"Highlights of the 1999 IHOU" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 1, 2000

"A Substandard Life Brokerage Agent's Perspective on Underwriting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 16, No. 1, 2000

"Liquid Gold: What Urine Can Do for You" NAILBA Magazine, Winter 2000, with Kip Whitefield

"Highlights of the 1999 CIU" On The Risk - Journal of the Academy of Life Underwriting, Vol. 15, No. 4, 1999

"Highlights of the 2nd International Underwriting Congress" On The Risk - Journal of the Academy of Life Underwriting, Vol. 15, No. 3, 1999

"Highlights of the 1999 HOLUA Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 15, No. 3, 1999

"Predicting Motor Vehicle Accidents Among the Elderly" Underwriter Alert, Vol. 9, No. 2, 1999

"Risk Factors for Elder Abuse" Journal of Insurance Medicine, Vol. 31, No. 1, 1999, pp 13-20

"Insurance Securitization" On The Risk - Journal of the Academy of Life Underwriting, Vol. 15, No. 1, 1999

"Highlights of the 1998 HOLUA: Capitol Crossfire" On The Risk - Journal of the Academy of Life Underwriting, Vol. 14, No. 3, 1998

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"DNA Typing for Individual Identification" On The Risk - Journal of the Academy of Life Underwriting, Vol. 14, No. 2, 1998

"DNA Typing for Individual Identification" FALU Paper, 1998

Highlights of the 1997 IHOU: Remember Our Mission" On The Risk - Journal of the Academy of Life Underwriting, Vol. 14, No. 1, 1998

"Underwriting and Risk Classification" Society of Actuaries' International Section News, No. 15, February 1998, with Chris Cook

"Highlights of the 1997 CIU: Reach for the Top" On The Risk - Journal of the Academy of Life Underwriting, Vol. 13, No. 4, 1997

"Highlights of the 1997 HOLUA: The Heat Is On" On The Risk - Journal of the Academy of Life Underwriting, Vol. 13, No. 3, 1997

"First International Underwriting Congress" Society of Actuaries' International Section News, No. 14, August 1997, with Chris Cook

"Highlights of the 1997 International Underwriting Congress" On The Risk - Journal of the Academy of Life Underwriting, Vol. 13, No. 2, with Chris Cook

"International Outlook: Highlights of the 1997 International Underwriting Congress" Resources, Vol. 17, No. 6 Supplement, 1997, with Chris Cook

"Highlights of the 1996 IHOU: Building Bridges" On The Risk - Journal of the Academy of Life Underwriting, Vol. 13, No. 1, 1997

"Medical Studies: Where Do They Come From? Transferring Medical Knowledge to Risk Selection" (Part 2 of 2)" Underwriter Alert, Vol. 6, No. 6, 1997

"Elevated GGT, Hypertension and Obesity" On The Risk - Journal of the Academy of Life Underwriting, Vol. 12, No. 4, 1996

"Clinical Trials and Population Studies (Part 1 of 2)" Underwriter Alert, Vol. 6, No. 5, 1996

"Highlights of the 1996 HOLUA: Navigating the Waves of Change" On The Risk - Journal of the Academy of Life Underwriting, Vol. 12, No. 3, 1996

"Underwriting Cultural Markets: Focus on Asian Indian Ethnicity" On The Risk - Journal of the Academy of Life Underwriting, Vol. 12, No. 3, 1996

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"Highlights of the Spring 1996 Risk Appraisal Forum" On The Risk - Journal of the Academy of Life Underwriting, Vol. 12, No. 2, 1996

"Tobacco and User/Abstainer Pricing" Product Development News Society of Actuaries, Issue 40, May 1996

"Underwriting Research Resources Among Life Insurers: A Survey" VFD Consulting, Inc. 1996

"Highlights of the 1995 ICLAM/AAIM Conference" On The Risk - Journal of the Academy of Life Underwriting, Vol. 12, No. 1, 1996, with John J. Krinik

"The Quest for Excellence: Highlights of the 1995 IHOU" On The Risk - Journal of the Academy of Life Underwriting, Vol. 12, No. 1, 1996

"Conference Review: 1995 Nucleic Acid-Based Technologies" On The Risk - Journal of the Academy of Life Underwriting, Vol. 11, No. 4, 1995

"Scaling New Peaks - Highlights of the 1995 HOLUA Annual Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 11, No. 3, 1995

"OTR Meeting Review: 1995 Society of Insurance Research Planning and Economics Conference" On The Risk - Journal of the Academy of Life Underwriting, Vol. 11, No. 3, 1995

"Tobacco and User/Abstainer Pricing" On The Risk - Journal of the Academy of Life Underwriting Vol. 11, No. 2, 1995

"OTR Meeting Review: Highlights of the 1994 IHOU" On The Risk - Journal of the Academy of Life Underwriting, Vol. 11, No. 1, 1995

"Underwriting Marital Violence" On The Risk - Journal of the Academy of Life Underwriting, Vol. 10, No. 5, 1994

"Highlights of the 1994 HOLUA Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 10, No. 4, 1994

"How Agents Can Cope With Reluctance to Sell DI" National Underwriter, Vol. 97, No. 49, 1993

"Both `Supply' and `Demand' Causing Sales Doldrums" National Underwriter, Vol. 97, No. 44, 1993

"Sailing for Success: Highlights of the 1993 IHOU Meeting" On The Risk - Journal of the Academy of Life Underwriting, Vol. 10, No. 2, 1994

Vera F. Dolan
Curriculum Vitae, continued

PUBLICATIONS - INSURANCE, continued

"Moderate Alcohol Intake and Reduced Mortality: Illusion or Preferred Risk?" On The Risk - Journal of the Academy of Life Underwriting, Vol. 10, No. 1, 1993

"Expanding Horizons: Highlights of the 1993 HOLUA" On The Risk - Journal of the Academy of Life Underwriting, Vol. 9. No. 4, 1993

"Underwriters Urged to Tap More Into Elderly Mkt." National Underwriter, Vol. 97, No. 23, 1993

Contributing Analyst, Medical Risks: Trends in Mortality by Age and Time Elapsed, Edward Lew and Jerzy Gajewski, Praeger Publishers, New York, 1990 (as Vera F. Kelly)

"Notes on Breast Cancer" On The Risk - Journal of the Academy of Life Underwriting, Vol. 4, No. 3, 1988 (as Vera F. Kelly)

"Underwriting Epilepsy" On The Risk - Journal of the Academy of Life Underwriting, Vol. 2, No. 4, 1986 (as Vera F. Kelly)

PUBLICATIONS – BUSINESS, HEALTH and OTHER

"Selling Our Children in the U.S.: An Interview with Russ Combs" The Voice: The Journal of the Domestic Violence Movement, Winter 2005

Contributor, After the Guns Fall Silent: The Enduring Legacy of Landmines, S. Roberts and J. Williams, Vietnam Veterans of America Foundation, Washington, D.C. 1995

Editor, The Politics of Information Systems, Paul Strassmann, Information Economics Press, New Canaan, CT, 1994

Invited Editorial Response, American Programmer, January, 1993

Editor-Producer, Win Some, Lose Some: My 40 Years in Corporate America, John Titsworth, Information Economics Press, New Canaan, CT, 1992

Editor, The Business Value of Computers, Paul Strassmann, Information Economics Press, New Canaan, CT, 1990

Editor, Information Payoff: The Transformation of Work in the Electronic Age, Paul Strassmann, Macmillan Free Press, NY, 1985 (as Vera F. Kelly)

"A Proportionate Mortality Study of an Oil, Chemical and Atomic Workers Local in Texas City, Texas" Annals of the New York Academy of Sciences, 381(1982): 54-61, with Reeve, Waxweiler, Thomas and Itaya (as Vera F. Kelly)

Vera F. Dolan
Curriculum Vitae, continued

PRESENTATIONS

"Isolated Hematuria: What Is the Risk?" Northern California Life Insurance Association, Walnut Creek, California, May 10, 2012

"Positive Cocaine Tests and Mortality" Northern California Life Insurance Association, Berkeley, California, September 17, 2009

"Actuaries and Public Health: From Analysis to Action" Society of Actuaries Health Spring Meeting, Los Angeles, California, May 28, 2008, with Grady Catterall and Dana Goldman

"Research Results: Potential Impact of a Pandemic on the U.S. Health Insurance System" Society of Actuaries Health Spring Meeting, Los Angeles, California, May 28, 2008, with Jim Toole and Jim Walbridge

"How the World Has Changed, and How Medical Underwriting Will Benefit Your Applicants and Your Companies' Bottom Line" Annual Product Development Actuary Symposium, Denver, Colorado, June 26, 2007, with Robert L. Stout

"Non-Underwritten Life Products – Do They Work?" Annual Product Development Actuary Symposium, Denver, Colorado, June 25, 2007, with Jack Simon

"Pandemics and Extreme Events" Actuarial Society of Greater New York Annual Meeting, New York City, New York, November 9, 2006, with Janet Carstens

"Underwriting Simplified Issue Life Insurance Products" Group Underwriting Association of America, San Diego, California, October 31, 2006, with Ernie Testa

"The Life Settlements Market" Society of Actuaries Annual Meeting, Chicago, Illinois, October 18, 2006, with Tony Duscio and Jay Vadiveloo

"Preparing for Extreme Events" Society of Actuaries Annual Meeting, Chicago, Illinois, October 18, 2006, with Janet Carstens and Max Rudolph

"Pandemic Exposure and Risk Management Strategies in Asia as Compared to the United States" Society of Actuaries Life Spring Meeting, Hollywood, Florida, May 24, 2006, with Ronald Klein

"Current Trends in Distribution Channels: New Underwriting for a New Millennium" Society of Actuaries Life Spring Meeting, New Orleans, Louisiana, May 23, 2005, with Jim McArdle and Ernie Testa

"Gynecologic and Obstetric Disorders" Institute of Caribbean Home Office Underwriters, Almond Beach Resort, Barbados, September 14, 2004

"Sleep Dysfunction and Disorders" Institute of Caribbean Home Office Underwriters, Almond Beach Resort, Barbados, September 13, 2004

SCIENTIFIC REPORTS

OPEN

Transmission of Ebola virus from pigs to non-human primates

Hana M. Weingartl[1,2], Carissa Embury-Hyatt[1], Charles Nfon[1], Anders Leung[3], Greg Smith[1] & Gary Kobinger[3,2]

[1]National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, 1015 Arlington St. Winnipeg, Manitoba, R3E 3M4, Canada, [2]Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada, [3]National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington St., Winnipeg, Manitoba, R3E 3R2, Canada.

SUBJECT AREAS:
VIROLOGY
PATHOGENS
PATHOLOGY
EXPERIMENTAL ORGANISMS

Received
25 April 2012

Accepted
28 September 2012

Published
15 November 2012

Correspondence and requests for materials should be addressed to H.M.W. (hana.weingartl@inspection.gc.ca) or G.K. (gary.kobinger@phac-aspc.gc.ca)

Ebola viruses (EBOV) cause often fatal hemorrhagic fever in several species of simian primates including human. While fruit bats are considered natural reservoir, involvement of other species in EBOV transmission is unclear. In 2009, Reston-EBOV was the first EBOV detected in swine with indicated transmission to humans. In-contact transmission of Zaire-EBOV (ZEBOV) between pigs was demonstrated experimentally. Here we show ZEBOV transmission from pigs to cynomolgus macaques without direct contact. Interestingly, transmission between macaques in similar housing conditions was never observed. Piglets inoculated oro-nasally with ZEBOV were transferred to the room housing macaques in an open inaccessible cage system. All macaques became infected. Infectious virus was detected in oro-nasal swabs of piglets, and in blood, swabs, and tissues of macaques. This is the first report of experimental interspecies virus transmission, with the macaques also used as a human surrogate. Our finding may influence prevention and control measures during EBOV outbreaks.

Ebola viruses belong to the family *Filoviridae*, genus *Ebolavirus*. Those endemic to Africa cause severe hemorrhagic fever with frequent fatal outcome in humans, great apes and several species of non-human primates (NHPs). Fruit bats are considered to be the natural reservoir for EBOV in Africa[1]. In 2009, the only non-African known species of EBOV, Reston Ebola virus (REBOV), was isolated from swine in Philippines, with antibodies against the virus detected in pig farmers[2,3]. However REBOV did not cause clinical signs in experimentally inoculated pigs[4]. In contrast to African species of EBOV, REBOV does not cause clinical symptoms in humans, although the infection may be fatal in cynomolgus macaques[5]. We have previously demonstrated that Zaire-EBOV (ZEBOV) can infect pigs, cause disease, and transmit to in-contact pigs[6]. While primates develop systemic infection associated with immune dysregulation resulting in severe hemorrhagic fever, the EBOV infection in swine affects mainly respiratory tract, implicating a potential for airborne transmission of ZEBOV[2,6]. Contact exposure is considered to be the most important route of infection with EBOV in primates[7], although there are reports suggesting or suspecting aerosol transmission of EBOV from NHP to NHP[8–10], or in humans based on epidemiological observations[11]. The present study was design to evaluate EBOV transmission from experimentally infected piglets to NHPs without direct contact.

Results

Six four-week old Landrace piglets (*Sus scrofa*) were oronasally inoculated with 10^6 $TCID_{50}$ of ZEBOV (Kikwit 95) per animal. The piglets were transferred to a separate room for the inoculations, and then moved back into the room containing four cynomolgus macaques. This age group was selected based on the previous observation of differences in severity of the disease in ZEBOV inoculated piglets[6] to ensure sufficient survival time of the piglets potentially needed for virus transmission, and to determine whether piglets without an overt clinical disease could transmit the virus. The macaques were housed in two levels of individual cages inside the pig pen, and separated from the piglets by wire barrier placed about 20 cm in front of the bottom cages to prevent direct contact between the two species. Bottom cages housing NHPs Nos. 07M and 20F were about 10 cm above the ground, top cages housing NHPs Nos. 34F and 51M were about 1.4 m above the ground. The NHP cages were located immediately to the side of the air exhaust system. The cubicle layout respective to the airflow (ten complete air exchanges per hour) in the room is schematically indicated in Supplemental Figure S1. During the husbandry, piglets were moved away from the cages and enclosed by the gate system. The floor was washed, taking care that the water is sprayed at low pressure and away from the NHP cages, to avoid any splashes into the bottom cages. Also the

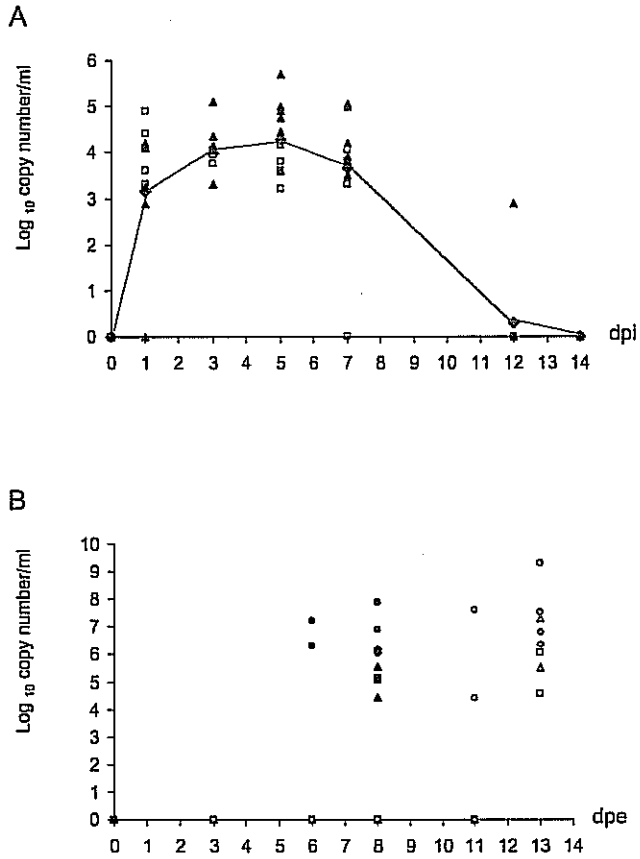


Figure 1 | Detection of EBOV RNA in swabs and blood. (A) Shedding in pigs. Squares represent the oral swabs and triangles illustrate the nasal swabs. Gray line with diamonds shows the general trend of the oro-nasal shedding. (B) Non-human primates: square markers represent the oral swabs, diamonds represent the rectal swabs, triangles represent the nasal swabs, circles represent blood samples. Gray markers-NHP No. 51M and 20F, black markers-NHP 07M and 34F. "dpi" (days post inoculation) and "dpe" (days post exposure) on the X axis are equivalent.

20 cm space between the wire barrier and the cages was cleaned separately with running water prior to proceeding with NHP cage cleaning. Both animal species were fed after the cleaning, providing new clean dishes for the macaques, with staff changing disposable outer gloves between procedures and animals. The design and size of the animal cubicle did not allow to distinguish whether the transmission was by aerosol, small or large droplets in the air, or droplets created during floor cleaning which landed inside the NHP cages (fomites). The husbandry flow during the sampling days was: cleaning, followed by sampling, then feeding, with staff changing disposable outer gloves between procedures and animals. Pigs and NHPs were sampled on alternative days except for day 3 post infection, when NHPs were sampled in the morning and the piglets in the afternoon.

Clinical signs and gross pathology in swine, following the inoculation with EBOV, were comparable to previous infection study in piglets of this age group[6]. Increase in respiratory rate (up to 80 breaths/min) and in rectal temperatures (40.2–40.5°C) was observed between 5 and 7 days post infection (dpi). All piglets apparently recovered from the disease by 9 dpi. Piglets Nos. 1, 2 and 4 were euthanized at 12 dpi, and piglets Nos. 3, 5 and 6 at 14 dpi, based on experimental schedule. Clinical scores and parameters are provided in the Supplementary Information (Supplemental Figure 2A, Supplemental Table 1). No significant lesions were observed at the necropsy. Microscopic lung lesions were focal and not extensive,

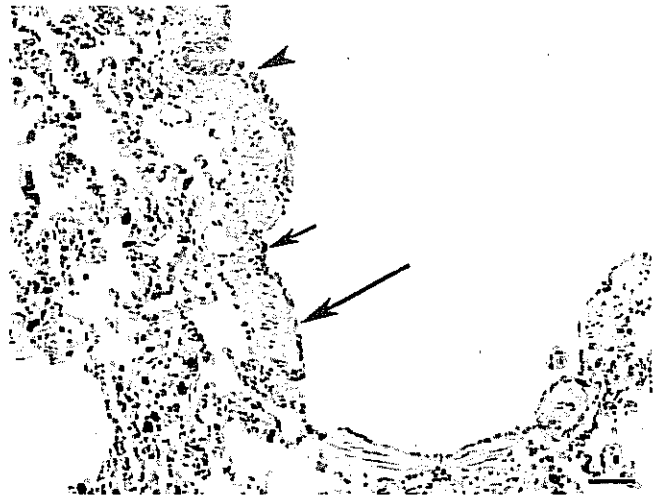


Figure 2 | Lungs, macaque No.34F. Segmental attenuation and loss of respiratory epithelium in the bronchiolar wall (large arrow) with some areas of the lungs relatively unaffected (arrowhead). Immunostaining for Ebola virus antigen was detected in occasional respiratory epithelial cells (small arrow) as well as within alveolar and septal macrophages. Bar=50 μm.

characterized by broncho-interstitial pneumonia with a lobular pattern, similar to those described in our previous report[6]. Virus antigen was detected by immunohistochemistry in three piglets (No. 2, 4, and week signal in No. 5), primarily within the areas of necrosis often adjacent to bronchioles (Supplemental Figure S3A). The presence of virus in the lung was confirmed by detection of EBOV RNA employing real-time RT-PCR targeting the L gene, and by virus isolation on Vero E6 cells for piglet No. 2 and No. 4. Virus isolation was also attempted from lung associated lymph nodes, based on detection of viral RNA, yielding one, successful isolation. Viral RNA was detected in submandibular lymph nodes of all piglets, and in the spleen and liver of two piglets. Low level of viremia based on RNA levels was detected in blood of four piglets at 5 and 7 dpi. EBOV RNA was detected in nasal and oral swabs of piglets from 1 dpi until 7 dpi, inclusively (Figure 1A), and from rectal swabs on day 1 and 5, but not at 3, 7 and 12 dpi (Supplemental Table 1). Viral isolation was attempted on all swabs. Out of 45 oral and nasal swabs positive by RT-PCR, 16 were positive on virus isolation, while two out of 11 RNA-positive rectal swabs tested positive for virus. Presence of EBOV RNA in cell culture supernatants from the isolates with observed CPE was confirmed by real time RT-PCR (Supplemental Table 1; Supplemental Table 2).

Air sampling was conducted on day 0, 3, 6, 8 and 11 post inoculation. Real time RT-PCR targeting the L gene detected viral RNA on days 6 and 8 post inoculation. Location in front of the bottom cages at about 75 cm above the floor was sampled in 30 min triplicates following husbandry, during the NHP sampling. Average values of 4.4 $\log_{10}$ copies/ml and 3.85 $\log_{10}$ copies/ml of the sampling buffer were detected at 6 and 8 dpi, respectively. Virus isolations were not successful, likely due to the sampling buffer composition (0.1% Tween 20).

All four NHPs (*Macaca fascularis*) were alert and in good apparent health until 7 days post exposure (dpe - corresponding to dpi of piglets) with ZEBOV. At 8 dpe, macaques 07M (bottom left cage) and 34F (upper right cage), housed in cages located within an air flow towards the exhaust system, were euthanized based on clinical signs typical for EBOV infection in NHPs. Both had petechial hemorrhages on the skin of the chest and along internal surfaces of the arms and legs. Macaques 51M and 20F were visually healthy until 12 dpe, when early clinical signs were noted, and both animals were

euthanized the next day (13 dpe). The NHPs were euthanized when convincing clinical signs typical for EBOV infection became apparent, preferably prior to the humane endpoint (Supplemental Figure S2B; Supplemental Table 1). Examination of internal organs at the necropsy exposed damages mainly to the lung (Supplemental Figure S4) and liver. Microscopic lesions and antigen distribution in the organs were similar to previous reports[12–14], except for the lesions and antigen distribution in lungs. Interstitial pneumonia was characterized by thickened and hypercellular alveolar septa due to infiltration by primarily macrophages (Supplemental Fig. 3B), with multifocal areas of alveolar hemorrhage and edema. EBOV antigen was detected extensively in alveolar and septal macrophages using double immunostaining (Supplemental Fig. 3C), as well as within pneumocytes and endothelial cells. Viral antigen was also observed within bronchiolar epithelial cells with adjacent segmental loss of epithelial cells (Figure 2.) and within respiratory epithelial cells of the trachea. The pattern of lesions and immunostaining for EBOV antigen in lungs suggests infection of the lungs both, via respiratory epithelium and due to viremic spread of the virus.

There was a remarkable difference in the type and quantity of cells infiltrating the lungs between the macaques and the pigs, although viral antigen was detected only in alveolar macrophages of both species. Monocytes/macrophages were essentially the only leukocyte type infiltrating the lungs in non-human primates, while large quantities of non-infected lymphocytes were recruited into the pig lungs. This phenomenon can be linked to different clinical picture in the two animal species: respiratory distress in pigs (severe in a specific age group[6]) versus systemic disease with no major respiratory signs in NHPs. It will be important to identify differences and similarities in ZEBOV-induced pathogenesis and pathology between the two species in future studies.

Infection of the NHPs with ZEBOV was confirmed by detection of viral RNA (real time RT-PCR targeting the L gene), and in all samples collected at euthanasia by virus isolation. The first detection of ZEBOV RNA was in the blood of NHPs 34F and 07M at 6 dpe, with virus isolation from macaque 07M. This was followed by ZEBOV RNA detection in nasal, oral and rectal swabs from the same NHPs at 8 dpe (Figure 1B). A similar pattern was observed for macaques 51M and 20F, starting at 11 dpe with detection of RNA in blood and virus isolation from animal 20F, followed by RNA and virus detection in swabs at 13 dpi. Detection of viral RNA and infectious virus in blood, swabs and tissues of the macaques (summarized in Supplemental Table 4) confirmed systemic spread of the virus. Whole genome sequencing performed on virus nucleic acid from selected swab and lung samples from pigs and NHPs confirmed identity of the virus.

Discussion

Pigs were the source of ZEBOV at a time of infection of NHPs euthanized at 8 dpe (07M and 34F) since shedding from the macaques was not detected at dpe 3 or 6. NHPs euthanized at 13 dpe (20F, 51M) could have contracted ZEBOV from the environment contaminated by either species, considering previous reports on development of disease following aerosol exposure[10], or other inoculation routes[5,15,16], although pigs can generate infectious short range large aerosol droplets more efficiently then other species[17]. We have also never observed transmission of EBOV from infected to naive macaques, including in an experiment employing the same cage setting as in the current study, where three NHPs intramuscularly inoculated with EBOV did not transmit the virus to one naive NHP for 28 days, the duration of the protocol. During another study, three EBOV infected NHPs cohabiting with 10 naive NHPs in adjacent cage systems did not transmit the virus to naive animals for 28 days (unpublished data). The exact route of infection of the NHPs is impossible to discern with certitude because they were euthanized at a time when EBOV had already spread systemically. However, the segmental attenuation and loss of bronchiolar epithelium and the presence of Ebola virus antigen in some of the respiratory epithelial cells in the lungs of all macaques suggest that the airways were one of the routes involved in the acquisition of infection, consistent with previous reports[9,10]. Other routes of inoculation generally did not lead to lesions in the respiratory tract comparable to those observed in this study[12,13].

Under conditions of the current study, transmission of ZEBOV could have occurred either by inhalation (of aerosol or larger droplets), and/or droplet inoculation of eyes and mucosal surfaces and/or by fomites due to droplets generated during the cleaning of the room. Infection of all four macaques in an environment, preventing direct contact between the two species and between the macaques themselves, supports the concept of airborne transmission.

It is of interest, that the first macaques to become infected were housed in cages located directly within the main airflow to the air exhaust system. The experimental setting of the present study could not quantify the relative contribution of aerosol, small and large droplets in the air, and droplets landing inside the NHP cages (fomites) to EBOV transmission between pigs and macaques. These parameters will need to be investigated using an experimental approach specifically designed to address this question.

The present study provides evidence that infected pigs can efficiently transmit ZEBOV to NHPs in conditions resembling farm setting. Our findings support the hypothesis that airborne transmission may contribute to ZEBOV spread, specifically from pigs to primates, and may need to be considered in assessing transmission from animals to humans in general. The present experimental findings would explain REBOV seropositivity of pig farmers in Philippines[2,3] that were not involved in slaughtering or had no known contact with contaminated pig tissues. The results of this study also raise a possibility that wild or domestic pigs may be a natural (non-reservoir) host for EBOV participating in the EBOV transmission to other species in sub-Saharan Africa.

Methods

Virus. ZEBOV strain Kikwit 95 was produced on VERO E6 cells in minimal essential medium (MEM) supplemented with 2% fetal bovine serum and antibiotics (Penicillin/Streptomycin). Virus titers were determined by standard $TCID_{50}$ and/or immunoplaque assays on VERO E6 cells. Procedures for the production and propagation of ZEBOV and all subsequent experiments involving infectious materials were performed in the Containment Level (CL) 4 facilities of the Canadian Science Center for Human and Animal Health (CSCHAH).

Animal experiments. Four cynomolgus macaques were acclimatized in the BSL4 animal facility for two weeks, and housed in the same room for one week prior to the swine inoculation. The macaques were housed in two levels of individual cages inside the pig pen, and separated from the piglets by wire barrier placed about 15 cm in front of the cages to prevent direct contact between the two species. Bottom cages housing NHPs Nos. 07M and 20F were about 20cm above the ground, while top cages housing NHPs Nos. 34F and 51M were about 1.4 m above the ground. The NHP were sampled at 3 and 6 dpi (nasal, oral rectal swabs, blood) as per experimental schedule. Two macaques were euthanized for humane reasons at 8 days post exposure (dpe), and all animals were sampled at that time. Two remaining NHPs were in addition sampled at 11 dpe, and at13 dpe when they were euthanized. The animals were euthanized when typical clinical signs of Ebola infection became apparent, if possible prior to reaching the humane endpoint. Lung, lung associated lymph nodes, liver, spleen and intestine were collected at the necropsy.

Pigs (breed Landrace) were obtained from a high health status herd operated by a recognized commercial supplier in Manitoba, Canada. Three-week old piglets, designated as animal No. 1–6, were acclimatized for seven days prior to the inoculation in an animal cubicle already housing the non-human primates. The six piglets were inoculated oro-nasally with 2 ml of 10^6 $TCID_{50}$ total per animal (0.5 ml per each nostril and 1 ml orally) in a room adjacent to the BSL4 animal cubicle and subsequently housed in proximity to cages with four non-human primates (NHP). Swine rectal temperatures were taken during the sampling performed under anesthesia on days 0, 1, 3, 5, 7, 12 and 14, when blood and rectal, oral and nasal swabs were collected. Three piglets were euthanized on day 12 post inoculation (no. 1M, 2M. 4F), and three on day 14 (3M, 5F, 6F), as per experimental schedule. Muscle, lung, liver, spleen, trachea, and submandibular, lung associated and mesenteric lymph nodes were collected at necropsy.

All animal manipulations were performed under CL4 conditions and followed Animal Use Document No. CSCHAH AUD# C-11-004 approved by the Animal Care

Committee of the Canadian Science Centre for Human and Animal Health, according to and following the guidelines of the Canadian Council on Animal Care.

Virus isolation. Swabs collected into 1 ml of cMEM, blood, and tissues homogenized in MEM using a bead mill homogenizer according to the manufacturer's protocol (Tissue Lyser, Qiagen) were used for virus isolation and real time RT-PCR analysis. All NHP samples and swine rectal swabs were plated in 10-fold serial dilutions of supernatant on Vero E6 cells with six replicates per dilution. At 72–96 h post-infection the plates were scored for cytopathic effect (CPE) and $TCID_{50}$ virus titers were calculated using the Reed and Muench method. Swine rectal swabs had to be however carried over onto replica plates for three passages prior to reading the CPE. Swine nasal and oral swabs, blood and tissues were first analyzed by real time RT-PCR targeting the ZEBOV L gene, followed by virus isolation on Vero E6 cells in P6 plates on selected samples.

Virus RNA detection. NHP samples: Total RNA was isolated from tissues preserved and homogenized in RNA later employing the RNeasy Mini Kit (QIAGEN). RNA from nasal washes and swabs was isolated using the QIAamp Viral RNA Mini Kit (QIAGEN, GmbH).

Swine samples: RNA was isolated using Tripure Reagent (Roche Applied Science) according to the manufacturer's recommendations from swabs, blood or 10% w/v tissue homogenates in cMEM. One-Step real-time RT-PCR was carried out using following primers and probe:

ZebovForward -CAGCCAGCAATTTCTTCCAT;
ZebovReverse- TTTCGGTTGCTGTTTCTGTG;
ZebovProbe FAM-ATCATTGGCGTACTGGAGGAGCAG-NFQ.

Armoured enterovirus RNA (Asuragen) was used as external extraction/reaction control. Quantitect Reverse Transcriptase Real-time PCR kit (Qiagen) was employed for the PCR reactions according to the manufacturer's specifications. Reaction conditions for the RT-PCR were as follows: 50°C for 30 minutes; 95°C for 15 minutes; 45 cycles of 95°C for 15 seconds followed by 60°C for 45 seconds. The samples were run on the Rotor-Gene 6000 (Qiagen) or on the the LightCycler 480 (Roche Applied Science). Copy numbers were determined based on the L-gene Ebola plasmid standard control curve. Cut off value for samples to be considered positive were 3 log_{10} copies/ml (Rotorgene) or 3.15 log_{10} copies/ml (LightCycler 480).

Air sampling. The air was sampled using BioCapture 650 Air Sampler (FLIR, Arlington, VA) on days 0, 3, 6, 8 and 11 post inoculation of the piglets. The air sampling started after husbandry, concurrent to NHP sampling, later in the morning before noon. Location in front of the bottom cages at about 75 cm above the floor was sampled in 30 min triplicates. The collection took place over a span of about two hours in total (three 30 min collection times with changes of cartridges in between). The air sampler device collects particles by bubbling the air through a pre-loaded buffer (0.74% Tris/0.1 Tween 20) provided in a sealed cartridge by the manufacturer. This solution is not optimal for recovery of live enveloped viruses, and virus isolation attempts were unsuccessful. ZEBOV RNA was detected by real time RT-PCR targeting the L gene.

EBOV sequencing. Viral RNA previously extracted for real time PCR was sequenced by first generating cDNA with the use of Omniscript reverse transcriptase (Qiagen) and random hexamers along with specific EBOV primers followed by PCR with iProof high fidelity DNA polymerase (Bio-Rad) with specific primers (available upon request). DNA sequencing was carried out using the 3730xl DNA Analyzer (ABI).

Histology and immunohistochemistry. Tissues were fixed in 10% neutral phosphate buffered formalin, paraffin embedded using standard procedures, sectioned at 5 m, and stained with hematoxylin and eosin (HE) for histopathologic examination. Detection of viral antigen was performed using A 1:2000 dilution of rabbit polyclonal anti-ZEBOV VP40 antibody as described previously[4]. Identification of macrophages in the lungs was performed by immunostaining for the macrophage/monocyte marker L1 using Clone Mac387 (Dako, USA) primary antibodies. The tissue sections were quenched for 10 minutes in aqueous 3% hydrogen peroxide, prior to retrieval of epitopes using high pH AR10 (BioGenex, CA) in a BioCare Medical Decloaking Chamber. Antibody Clone Mac 387 was applied for 10 minutes at a dilution of 1:3200, and visualized using an AP-polymer kit, Mach 4 Universal (BioCare Medical, CA) for 30 minutes, and reacted with Vulcan Fast Red (BioCare Medical, CA) substrate. For the Mac387/Ebola double stain, antibody Clone Mac 387 was applied for 10 minutes at a dilution of 1:3200, and visualized using a multilink horseradish peroxidase labeled kit, Super Sensitive Link-Label IHC Detection System (BioGenex, CA), reacted with the chromogen diaminobenzidine (DAB). The sections were then incubated with a denaturing solution (1 part A, 3 parts B, BioCare Medical, CA) for 5 minutes, pretreated with proteinase K enzyme for 10 minutes, and rabit polyclonal anti-Ebola Zaire VP40 antibody was applied to the sections at a 1:2,000 dilution for one hour. The anti-EBOV antibody was visualized using an AP-polymer kit, Mach 4 Universal (BioCare Medical, CA) for 30 minutes and reacted with Vulcan Fast Red (BioCare Medical, CA) substrate. All sections are counterstained with Gill's hematoxylin.

1. Leroy, E. M. *et al.* Fruit bats as reservoirs of Ebola virus. *Nature* **438**, 575–576 (2005).
2. Barette, R. W. *et al.* Discovery of swine as a host for the *Reston* ebolavirus. *Science* **325**, 204–206 (2009).
3. WHO, Ebola Reston in pigs and humans, Philippines. *Weekly Epidemiological Record* **7**, 47–50 (2009).
4. Marsh, G. A. *et al.* Ebola Reston virus infection in pigs: clinical significance and transmission potential. *J. Infect. Dis.* **204** (Suppl.3), S804–S809 (2011).
5. Jahrling, P. B. *et al.* Experimental infection of cynomolgus macaques with Ebola-Reston filoviruses from the 1989–1990 U.S. epizootic. *Arch Virol Suppl* **11**, 115–134 (1996).
6. Kobinger, G. P. *et al.* Replication, pathogenicity, shedding, and transmission of Zaire ebolavirus in pigs. *J. Infect. Dis.* **204**, 200–208 (2011).
7. Feldmann, H. & Geisbert, T. W. Ebola haemorrhagic fever. *The Lancet* **377**, 849–862 (2011).
8. Dalgard, D. W. *et al.* Combined simian hemorrhagic fever and Ebola virus infection in cynomolgus monkeys. *Lab Anim. Sci.* **42**, 152–157 (1992).
9. Jaax, N. *et al.* Transmission of Ebola virus (Zaire strain) to uninfected control monkeys in a biocontainment laboratory. *The Lancet* **346**, 1669–1671 (1995).
10. Johnson, E., Jaax, N., White, J. & Jahrling, P. Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. *Int. J. Exp. Path.* **76**, 227–236 (1995).
11. Roels, T. H. *et al.* Ebola hemorrhagic fever, Kikwit, Democratic Republic of the Congo (1995: Risk factors for patients without a reported exposure. *J. Infect. Dis.* **179** (Suppl.1), S92- S97 (1999).
12. Baskerville, A., Bowen, E. T., Platt, G. S., McArdell, L. B. & Simpson, D. I. The pathology of experimental Ebola virus infection in monkeys. *J. Pathol.* **125**, 131–138 (1978).
13. Jaax, N. K. *et al.* Lethal experimental infection of rhesus monkeys with Ebola-Zaire (Mayinga) virus by the oral and conjunctival route of exposure. *Arch. Pathol. Lab. Med.* **120**, 140–55 (1996).
14. Larsen, T. *et al.* Pathologic findings associated with delayed death in nonhuman primates experimentally infected with Zaire Ebola virus. *J. Infect. Dis.* **196** Suppl 2, S323–S328 (2007).
15. Geisbert, T. W. *et al.* Vesicular stomatitis virus-based vaccines protect nonhuman primates against aerosol challenge with Ebola and Marburg viruses. *Vaccine* **26**, 6894–6900 (2008).
16. Geisbert, T. W. *et al.* Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* **375**, 1896-905 (2010).
17. Donaldson, A. I. & Alexandersen S. Predicting the spread of foot and mouth disease by airborne virus. *Rev Sci Tech OIE* **21**, 569–575 (2002).

Acknowledgement

We would like to thank Dr. Melanie van der Loop, Kevin Tierney, and Gary Wong for the assistance with animal care, to Peter Marszal, Jill Graham and Brad Collignon for the technical assistance, and to Dr. Soren Alexandersen for the critical review of the manuscript. The project was supported by CFIA and PHAC, with funding provided from the CRTI Cluster Activity fund CRTI-3780-2011-30va-17.

Author contributions

H.M.W. and G.K. conceived the study, design experiments, performed the animal experiments, analyzed and interpreted data, and wrote the manuscript. C.E-H. provided analysis of histopathology and data interpretation; A.L., G.S. and C.N. performed *in vitro* experiments and analyzed related data.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Weingartl, H.M. *et al.* Transmission of Ebola virus from pigs to non-human primates. *Sci. Rep.* **2**, 811; DOI:10.1038/srep00811 (2012).

Oxford Journals Medicine & Health The Journal of Infectious Diseases Volume 179, Issue Supplement 1 Pp. S92-S97.

ONE IS HERE

Journal of Infectious Diseases
jid.oxfordjournals.org

J Infect Dis. (1999) 179 (Supplement 1): S92–S97. doi: 10.1086/514286

Ebola Hemorrhagic Fever, Kikwit, Democratic Republic of the Congo, 1995: Risk Factors for Patients without a Reported Exposure

T. H. Roels, A. S. Bloom, J. Buffington*, G. L. Muhungu, W. R. Mac Kenzie, A. S. Khan, R. Ndambi, D. L. Noah, H. R. Rolka, C. J. Peters and T. G. Ksiazek
+ Author Affiliations

+ Author Notes

Correspondence: Dr. Thierry H. Roels, CDC/HIV Projet RETRO-CI, Côte d'Ivoire 2010, State Dept., Washington, DC 20521-2010 (tbr6@cdc.gov).

Abstract

In 1995, 316 people became ill with Ebola hemorrhagic fever (EHF) in Kikwit, Democratic Republic of the Congo. The exposure source was not reported for 55 patients (17%) at the start of this investigation, and it remained unknown for 12 patients after extensive epidemiologic evaluation. Both admission to a hospital and visiting a person with fever and bleeding were risk factors associated with infection. Nineteen patients appeared to have been exposed while visiting someone with suspected EHF, although they did not provide care. Fourteen of the 19 reported touching the patient with suspected EHF; 5 reported that they had no physical contact. Although close contact while caring for an infected person was probably the major route of transmission in this and previous EHF outbreaks, the virus may have been transmitted by touch, droplet, airborne particle, or fomite; thus, expansion of the use of barrier techniques to include casual contacts might prevent or mitigate future epidemics.

In 1995, 316 people became ill with Ebola (EBO) hemorrhagic fever (EHF) in Kikwit, Democratic Republic of the Congo (DRC). Prior to this, only three major outbreaks of EHF had been reported to the international medical community. The first two outbreaks occurred in 1976, when concurrent epidemics caused by 2 distinct virus subtypes [1–4] were reported in northern DRC (318 cases; case-fatality rate, 88%) [5] and the southern Sudanese towns of Maridi and Nzara (284 cases; case-fatality rate, 53%) [6]. In 1979, a smaller (n = 34) EHF outbreak recurred in Nzara [7]. Isolated reports of EHF were subsequently noted in DRC (1977, 4 cases) and Côte d'Ivoire (1994, 1 case) [8, 9]. In these outbreaks, provision of nursing care by health workers and household members was a risk factor for infection, as were working in a specific cotton factory, admission to a hospital, receipt of an injection, and assistance during birth by an infected woman [10].

Epidemiologists identified primary cases (the person who introduced the disease into the family or group under study [11]) in all of the outbreaks noted above. However, the source of infection (reservoir), mode of transmission, and risk factors associated with infection in patients with primary cases could not be determined. In all but the 1976 Nazara outbreak, field studies of patients with secondary cases established either contact with an EHF-infected person or another risk factor for

infection in patients without such contacts. The likely spread of infection in the community could therefore be traced, and the entire epidemic attributed to the patient with the primary case. In the 1976 Nzara outbreak, investigators were unable to document contact with an infected person or any other previously identified risk factor for 14 patients (21%) [6]. Similarly, in Kikwit, 55 (17%) of the 316 persons with likely EHF had no reported contact with another infected person during an initial assessment. These 55 individuals were the focus of a detailed epidemiologic investigation conducted to determine if a source of exposure could be found when systematic investigation procedures were followed; if social customs, occupational activities, or dietary habits might explain infection in patients for whom no source of infection was identified; and if specific risk factors for transmission existed among patients for whom an exposure source was identified.

Methods

During the 1995 Kikwit outbreak of EHF, surveillance to identify cases included the following passive and active components [12]: centralized death registry, retrospective and prospective review of clinic and hospital records, door-to-door case finding, and rumor registry (i.e., hearsay evidence of persons who had symptoms compatible with EHF).

All cases identified via these methods were initially investigated by interviewers using a short, standard questionnaire that categorized cases into those with or without a reported source of exposure as determined by a history of physical contact with an infected person.

A probable case of EHF was defined as an illness characterized by the following signs and symptoms in a resident of or visitor to the Bandundu region of DRC between January and August 1995: fever and unexplained hemorrhage (e.g., gingival bleeding, sub-conjunctival bleeding, petechia, purpura, melena, and hematemesis); or fever and previous contact with another EHF infected person; or fever accompanied by (any three) headache, nausea, vomiting, anorexia, intense fatigue, abdominal pain, myalgia, arthralgia, dysphagia, dyspnea, or hiccups.

A confirmed case of EHF was defined by the detection of EBO antigen or antibody, using ELISA, or the detection of viral RNA, using reverse transcription-polymerase chain reaction in a person who had signs or symptoms suggestive of EHF [13, 14].

These surveillance case definitions were used to screen eligible participants for this study; only those patients without a reported source of exposure were included. If a patient was deceased, a household member or a close relative was interviewed about the patient. These surrogates were chosen using a hierarchical preference system. The first surrogates chosen were individuals residing in the same household (i.e., sharing the same cooking fire) and who knew the patient well (e.g., the deceased patient's spouse or oldest child). Next, individuals from a different household who knew the patient well (e.g., the deceased patient's parents or grand-parents) were chosen. The last surrogates chosen were individuals residing on the same parcel of land as the patient (e.g., the deceased patient's landlord or closest neighbor).

After each patient (or surrogate) interview, sex- and age-matched (±10 years) control subjects were sought, using a systematic selection process. Three control subjects were selected for each patient enrolled in the study: 1 family control and 2 neighborhood controls. A family control was defined as a person who resided in the same household as the patient and had not had signs or symptoms suggestive of EHF between 1 January 1995 and the interview date. Because some families had many members who had died during the epidemic, it was not always possible to designate an individual of the same sex and age group to serve as the household control. Therefore, we developed a hierarchical system of identifying

family controls. First, we looked for an individual of the same sex and within 10 years of the patient's age. If there were no family members that fit those criteria, we looked for an individual of the opposite sex and within 10 years of the patient's age. If that failed, we looked for an individual of the same sex and > 10 years older or younger than the patient, and last, we looked for an individual of the opposite sex and >10 years older or younger than the patient.

Neighborhood controls were selected from among those persons who resided at least 5 houses away from patients to ensure that neighbors were from the same area but did not share the same cooking fire as the patient. In an effort to exclude control subjects who may have been infected with EHF between 1 January 1995 and the time of the interview, all potential control participants were screened using a structured questionnaire; those with signs and symptoms suggestive of EHF during this period were offered serologic testing to confirm or exclude the diagnosis, but they were not enrolled in the study.

Interviews of patients and control subjects were identical in format, order, and content. In addition to demographic information, the survey included questions regarding potential risk factors for infection, dietary habits, social customs, and occupational activities during the 3 weeks before onset of illness in patients and the same 3-week interval for matched control subjects. The potential risk factors for transmission of EHF that were evaluated in the interview included admission to a hospital for a preceding illness, receipt of an injection, working in a health care facility, attending a funeral, preparing a body for burial, visiting a friend with fever and bleeding, and physical contact outside the home (e.g., market, workplace) with a person who had fever and bleeding.

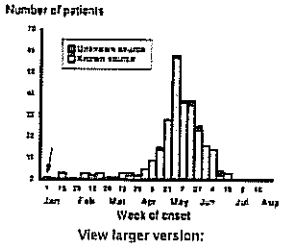
Univariate crude conditional maximum likelihood estimates of odds ratios (OR) and exact 95% confidence intervals (CI) were determined for each potential risk factor, using Epi Info software (version 6.02; USD, Stone Mountain, GA). For the multivariate analyses in this matched case-control study, exact conditional logistic regression (LogXact; Cytel Software, Cambridge, MA) was used. A multivariate model was constructed, which included univariate analysis risk factors with a significance level of $P \leq .05$. Interactions between variables were explored; the effect measures were not significant. The backwards stepwise elimination procedure was applied.

Results

On the basis of a review of all initial surveillance information, 55 of 316 patients had no reported source of exposure at the beginning of the investigation. Interviews were conducted with 44 (80%) of the 55 eligible patients (8 patients could not be located, and 3 refused to participate). Four patients had survived the illness and were able to participate in the survey. Surrogates provided information for the remaining 40 patients (91%). Serum specimens were obtained from 11 patients (25%); all were positive for EBO virus. The mean ages of the 44 patients and 132 control subjects was 37 years. Eighteen patients (41%) and 72 control subjects (55%) were female ($P = 2$).

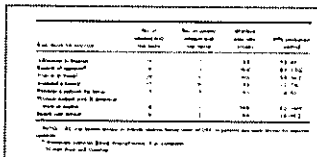
In-depth interviews identified potential risk factors for 32 (73%) of the 44 patients. Seven risk factors were statistically significant by univariate matched analysis (table 1). After we applied a multivariate logistic regression, two risk factors remained statistically significant in the final model: hospitalization for an unrelated illness before onset of EHF and visiting a person with fever and bleeding.

Figure 1
Onset date of Ebola hemorrhagic fever in 273 patients, by known source of



View larger version:
In this page In a new window
Download as PowerPoint Slide

exposure to infection (Kikwit, Democratic Republic of the Congo, 1995). Onset date was missing for 32 patients, and source was not assessed for 11 (3 refused to participate, 8 could not be located).



View larger version:
In this page In a new window
Download as PowerPoint Slide

Table 1
Matched odds ratios (crude) of univariate analysis of a matched case–control (1:3) study comparing risk factors for infection in 44 patients without reported source of exposure to Ebola virus at the start of this investigation and 132 controls during the Ebola hemorrhagic fever (EHF) outbreak (Kikwit, Democratic Republic of the Congo, 1995).

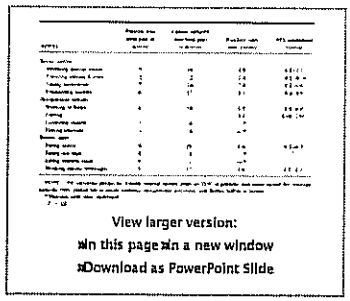
Fifteen (35%) of 43 patients (1/43 did not provide a response) reported admission to a health care center for an unrelated illness during the 3 weeks preceding onset of EHF; 7 (5%) of 132 control subjects reported hospital admission during the same time period (matched OR = 12; 95% CI = 2.7–76.8). At least 7 of the 15 patients had been admitted to Kikwit General Hospital (the major community hospital) for the previous illness, the first on 28 April 1995. The diagnoses at the time of hospital admission included malaria, tuberculosis, gastrointestinal disorders, and pregnancy-related complications. Nine patients (60%) were reported to have received an injection during their hospitalization.

The second independent risk factor for infection was visiting a person with fever and bleeding during the 3-week period before onset of EHF. Twenty-three (58%) of 40 patients and 11 (8%) of 131 control subjects were reported to have visited a friend or relative with fever and bleeding during the 3 weeks preceding onset of EHF (matched OR = 10; 95% CI = 3.2–38.9); information was not available for 4 patients and 1 control. The matched design of the study precluded analysis of other potential risk factors related to the visit (the few [11] control subjects who reported visiting a sick friend were not matched to patients who reported similar visits).

The probable source of exposure was identified for 32 (73%) of the 44 patients. Seventeen had visited an ill friend or relative with symptoms suggestive of EHF, 9 had been admitted to a health center in the 3 weeks preceding onset of EHF symptoms, and 6 had both risk factors. Of the 23 who had visited an ill friend or relative with symptoms suggestive of EHF, 4 (17%) resided in the same household as the ill patient and were their caregivers, 14 reported touching the ill patient, and 5 visited without touching the patient.

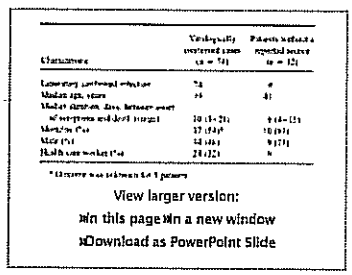
Information regarding the 12 patients who did not have a reported source of exposure and their matched controls was used to explore the role of social customs, occupational activities, and dietary habits in the acquisition of EHF infection (table 2). The univariate analysis did not identify any specific custom, occupation, or dietary factor that significantly increased the risk of infection. Although these 12 patients lacked serologic confirmation of EBO infection, their median age and duration of illness prior to death did not differ substantially from those of patients who had

virologically confirmed EHF (table 3), and their high mortality rate was consistent with rates reported in previous outbreaks of infection with the Zaire subtype of EBO.



View larger version:
»In this page »In a new window
»Download as PowerPoint Slide

Table 2
Results of a matched case–control (1:3) study comparing social customs, occupational activities, and dietary habits in 12 patients without identified source of exposure to Ebola hemorrhagic fever (EHF) at study completion and their matched controls (Kikwit, Democratic Republic of the Congo, 1995).



View larger version:
»In this page »In a new window
»Download as PowerPoint Slide

Table 3
Characteristics of 74 patients with virologically confirmed infection with Ebola hemorrhagic fever (EHF) and 12 patients without identified source for exposure to EHF at study completion (Kikwit, Democratic Republic of the Congo, 1995).

Figure 1 (epidemic curve) depicts the epidemic by week of onset for the 273 patients for whom date of onset and information on exposure source was available. Dates of illness onset were available for 11 of the 12 patients who had an unknown source of exposure; no clustering was observed.

Discussion

The 1995 Kikwit epidemic was the fourth major outbreak of EHF reported and the first to occur in a large population center. At the start of this investigation, 55 patients did not have a reported source of exposure to EHF; at the end, only 12 patients had no reported source. Among these 12 patients, no behavioral, occupational, or dietary activity was associated with illness. Transmission via inanimate objects (e.g., patient clothing, food utensils) was not implicated in this epidemic and has never been proven in previous investigations.

Admission to a health care center for an illness during the 3 weeks preceding onset of EHF was a statistically significant risk factor for infection during the outbreak. It was not possible to identify individual risk factors during hospitalization or determine the exact mode of transmission within Kikwit hospitals, primarily because of the numerous opportunities for disease transmission in open, crowded wards and outpatient clinics (e.g., direct or indirect contact with other sick patients or nursing staff, bedpans, soiled linen or clothing, and contaminated equipment). Although indirect transmission of EBO virus via reuse of nonsterile needles and syringes was a major route of transmission during the EHF outbreak in DRC in 1976 [5], this study did not identify receipt of an injection as an independent risk factor.

The main transmission route of EBO virus is believed to be direct or indirect inoculation of mucous membranes. In Kikwit, as in previous outbreaks, it was customary for a close family member or friend to serve as the patient's caretaker, that is, to clean, feed, and assist the patient both at home

and in the hospital. Before recognition of EBO virus as the cause of this outbreak, care was administered without precautions to prevent exposure to infected blood, vomitus, urine, or stool. Almost inevitably, caregivers, particularly spouses of patients, became infected with the virus [15].

Of the 23 patients in our study who were subsequently determined to have had previous exposure to a case of EHF, 19 had merely visited another patient with EHF and were not involved in patient care. None reportedly had any contact with patient blood, feces, vomitus, urine, or saliva, although 14 reported touching a patient with EHF. Recent immunohistochemical examination of skin biopsy specimens from patients with EHF has demonstrated viral antigens in skin and sweat glands [16], supporting the hypothesis that EHF may have been transmitted to these individuals in Kikwit (and others in previous outbreaks) by brief, unnoticed, superficial contact with EHF-in-fected persons.

The transmission mode in the 5 patients who became infected without any physical contact remains enigmatic. However, animal experiments have documented transmission of EBO virus via noncontact routes. For example, both guinea pigs and monkeys have been infected experimentally with EBO virus by direct installation of drops into the eye and throat [17]. Transmission of EBO virus from experimentally infected monkeys to control monkeys in separate cages has also been documented [18]. Furthermore, airborne spread was suggested during the EBO epizootic outbreak in Reston, Virginia [19, 20, 21]. In a review, Peters et al. [22] concluded that although the major mode of interhuman transmission of hemorrhagic fevers is direct contact, transmission via large droplets, aerosolized particles, or fomites cannot be excluded. This may explain the mode of transmission in the 5 patients without reported physical contact.

Our investigation had several limitations. First, the team frequently had to rely on surrogates to provide answers for patients who had died. Responses by surrogates describing the type of contact between deceased patients and ill persons visited by the patient may have been inaccurate, particularly if the surrogate had not accompanied the patient. Second, the interviewers may have been more aggressive in attempting to establish potential contacts or risk factors for patients than for control subjects. Third, although serologic confirmation of all cases would have been preferable, only 11 of 44 cases provided sera for confirmation. Fourth, the interval between the period of interest and the date of the interview was long and may have resulted in inaccuracies. Fifth, our matched design made it impossible to identify specific risk factors during hospitalization or during a visit to a sick person.

In conclusion, we identified an exposure source for 32 of 44 patients for whom no source was originally reported. Of the 12 patients who did not have an identified exposure source, no sociologic, occupational, or dietary risk factors for illness were found. Direct person-to-person contact was the likely mode of transmission for most EHF cases during this outbreak. However, our findings suggest that other EHF transmission modes cannot be excluded and may account for infection in those individuals for whom no previously recognized mode of transmission could be documented.

Acknowledgments

We are indebted to the International Scientific Technical Committee and to our team of DRC field investigators: Rafael Kabongo, Augustine Matanzi, Marie-Therese Mevela, Theo Mayanga, Cyprien, Frank Nsasu Mboso, Christian Mpassy, and Sangibala N'Kumat.

Footnotes

Reprints: Special Pathogens Branch, Division of Viral and Rickettsial Diseases, NCID, Mailstop A-26, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA, 30333.

References

1. Feldmann H, Klenk HD, Sanchez A. Molecular biology and evolution of filoviruses. Arch Virol 1993;7(suppl):81–100.
CrossRef Google Scholar

2. Buchmeier MJ, DeFries RU, McCormick JB, Kiley MP. Comparative analysis of the structural polypeptides of Ebola virus from Sudan and Zaire. J Infect Dis 1983;147:276–81.
Abstract/FREE Full Text

3. Cox NJ, McCormick JB, Johnson KM, Kiley MP. Evidence for two subtypes of Ebola virus based on oligonucleotide mapping of RNA. J Infect Dis 1983;147:272–5.
Abstract/FREE Full Text

4. McCormick JB, Bauer SP, Elliot LH, Webb PA, Johnson KM. Biologic differences between strains of Ebola virus from Zaire and Sudan. J Infect Dis 1983;147:264–7.
Abstract/FREE Full Text

5.WHO/International Study Team. Ebola hemorrhagic fever in Zaire, 1976. Bull World Health Organ 1978;56:271–93.
Medline Web of Science

6.WHO/International Study Team. Ebola hemorrhagic fever in Sudan, 1976. World Health Organ 1978;56:247–70.

7. Baron RC, McCormick JB, Zubeir OA. Ebola virus disease in southern Sudan: hospital dissemination and intrafamilial spread. Bull World Health Organ 1983;62:997–1003.
Google Scholar

8. Heymann DL, Weisfeld JS, Webb PA, Johnson KM, Cairns T, Berquist H. Ebola hemorrhagic fever: Tandala, Zaire, 1977–78. J Infect Dis 1980;142:373–6.
Google Scholar

9. Le Guenno B, Formenty P, Wyers M, Gounon P, Walker F, Boesch C. Isolation and partial characterisation of a new strain of Ebola virus. Lancet 1995;345:1271–4.
CrossRef Medline Web of Science Google Scholar

10. Breman JG, Piot P, Johnson KM, et al. The epidemiology of Ebola hemorrhagic fever in Zaire, 1976. In: PattynSR, editor. Ebola virus infection: proceedings of an international colloquium on Ebola virus infection and other hemorrhagic fevers. Amsterdam: Elsevier/North Holland Biomedical Press; 1978.
Google Scholar

11. Last JM. A dictionary of epidemiology. New York: Oxford University Press; 1988.
Google Scholar

12. Khan AS, Tshioko FK, Heymann DL, et al. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. J Infect Dis 1999;179 suppl 1:S76–86.
Abstract/FREE Full Text

13. Kzlazek TG, West CP, Rollin PE, Jahrling PB, Peters CJ. ELISA for the detection of antibodies to Ebola viruses. J Infect Dis 1999;179 suppl 1:S192–8.
Abstract/FREE Full Text

14. Sanchez A, Kslazek TG, Rollin PE, et al. Detection and molecular characterization of Ebola viruses causing diseases in human and nonhuman primates. J Infect Dis 1999;179 suppl 1:S164–9.
Abstract/FREE Full Text

15. Dowell SF, Mukunu R, Klaszek TG, et al. Transmission of Ebola hemorrhagic fever: a study of risk factors in family members, Kikwit, Democratic Republic of the Congo, 1995. J Infect Dis 1999;179 suppl 1:S87–91.
Abstract/FREE Full Text

16. Zaki SR, Shieh WJ, Greer PW, et al. A novel immunohistochemical assay for detection of Ebola virus in skin: implications for diagnosis, spread, and surveillance of hemorrhagic fever. J Infect Dis 1999;179 suppl 1:S36–47.

CrossRef Medline Web of Science Google Scholar

17. Jaax NC, Davis KJ, Geisbert TJ, et al. Lethal experimental infection of Rhesus monkeys with Ebola-Zaire (Mayinga) virus by the oral and conjunctival route of exposure. Arch Pathol Lab Med 1996;120:140–55.
Medline Web of Science Google Scholar

18. Jaax N, Geisbert T, Jahrling P, et al. Natural transmission of Ebola virus (Zaire strain) to monkeys in a biocontainment laboratory. Lancet 1996;346:1669–71.
CrossRef Web of Science Google Scholar

19. Dalgard DW, Hardy RJ, Pearson SL, et al. Combined simian hemorrhagic fever and Ebola virus infection in cynomolgus monkeys. Lab Anim Sci 1992;42:152–7.
Medline Web of Science Google Scholar

20.Centers for Disease Control. Update: filovirus infection among persons with occupational exposure to nonhuman primates. MMWR 1990;39:266–7, 273.
Medline

21. Johnson E, Jaax N, White J, Jahrling P. Lethal experimental infection of rhesus monkeys by aerosolized Ebola virus. Int J Exp Pathol 1995;76:227–36.
Medline Web of Science Google Scholar

22. Peters CJ, Jahrling PB, Khan AS. Patients infected with high-hazard viruses: scientific basis for infection control. Arch Virol 1996;11(suppl):141–68.
Google Scholar

Articles citing this article

Fifty–Five Years of International Epidemic–Assistance Investigations Conducted by CDC's Disease Detectives
Am J Epidemiol (2011) 174 (suppl_11): S97–S112
»Abstract »Full Text (HTML) »Full Text (PDF)

Filovirus Outbreak Detection and Surveillance: Lessons From Bundibugyo
The Journal of Infectious Disease (2011) 204 (suppl_3): S761–S767
»Abstract »Full Text (HTML) »Full Text (PDF)

Research Ethics and International Epidemic Response: The Case of Ebola and Marburg Hemorrhagic Fevers
Public Health Ethics (2009) 2 (1): 7–29
»Abstract »Full Text (HTML) »Full Text (PDF)

"Filoviruses": a real pandemic threat?
EMBO Mol Med. (2009) 1 (1): 10–18
»Abstract »Full Text (HTML) »Full Text (PDF)

Providing Care and Facing Death: Nursing During Ebola Outbreaks in Central Africa
J Transcult Nurs (2005) 16 (4): 289–297
»Abstract »Full Text (PDF)

Long–Term Di… Surveillance… Republic… Congo: A Mo…
The Journal of In…
»Abstract »…

An Introducti…
The Journal of Infectious Disease (1999) 179 (Supplement_1): ix–xvi
Online »Full Text (HTML) »Full Text (PDF) 2-1899

SUPPLEMENT ARTICLE

Assessment of the Risk of Ebola Virus Transmission from Bodily Fluids and Fomites

Daniel G. Bausch,[1,2] Jonathan S. Towner,[2] Scott F. Dowell,[3] Felix Kaducu,[4] Matthew Lukwiya,[5,a] Anthony Sanchez,[2] Stuart T. Nichol,[2] Thomas G. Ksiazek,[2] and Pierre E. Rollin[2]

[1]Tulane School of Public Health and Tropical Medicine, New Orleans, Louisiana; [2]Special Pathogens Branch and [3]Coordinating Office for Global Health, Centers for Disease Control and Prevention, Atlanta, Georgia; [4]Gulu Regional Hospital and [5]St. Mary's Hospital Lacor, Gulu, Uganda

Although Ebola virus (EBOV) is transmitted by unprotected physical contact with infected persons, few data exist on which specific bodily fluids are infected or on the risk of fomite transmission. Therefore, we tested various clinical specimens from 26 laboratory-confirmed cases of Ebola hemorrhagic fever, as well as environmental specimens collected from an isolation ward, for the presence of EBOV. Virus was detected by culture and/or reverse-transcription polymerase chain reaction in 16 of 54 clinical specimens (including saliva, stool, semen, breast milk, tears, nasal blood, and a skin swab) and in 2 of 33 environmental specimens. We conclude that EBOV is shed in a wide variety of bodily fluids during the acute period of illness but that the risk of transmission from fomites in an isolation ward and from convalescent patients is low when currently recommended infection control guidelines for the viral hemorrhagic fevers are followed.

Ebola virus (EBOV; family *Filoviridae*, genus *Ebolavirus*, type species *Zaire ebolavirus*) circulates in sub-Saharan Africa, where it occasionally causes large outbreaks of severe hemorrhagic fever with high case fatality rates [1]. The natural reservoir remains unknown, although bats are suspected [2]. Because no effective vaccine or specific antiviral therapy is available for Ebola hemorrhagic fever (EHF), the primary control strategy relies on aggressive contact tracing and isolation of patients with suspected cases in specialized isolation wards [3].

Large outbreaks of EHF are usually driven by person-to-person transmission, with caregivers both at home and in hospitals being at particular risk [4]. Although direct contact with bodily fluids is considered to be the major risk factor [5–7], other than confirmation of EBOV in blood during acute illness, few data exist on which specific bodily fluids pose a risk and at what stages of infection. Furthermore, although extreme caution is recommended to prevent environmental contamination and exposure in isolation wards and detailed safety guidelines and protocols for decontamination have been developed [3], the role of fomites in the transmission of EBOV has not been explored. To better understand the precise modes of transmission, we sampled various clinical specimens from patients as well as from environmental surfaces in an isolation ward for EHF and analyzed them for the presence of EBOV.

METHODS

Clinical specimens. The study was conducted in the isolation ward at Gulu Regional Hospital during an outbreak of EHF (Sudan EBOV) in Gulu, Uganda, in 2000 [8]. The ward was divided into patients with "suspected EHF" and patients with "probable EHF" on the basis of the clinician's judgment with subsequent use of the laboratory data when available. The daily cleaning/decontamination procedure in the ward consisted of spraying a 0.5% bleach solution on the floors each

Potential conflicts of interest: none reported.

Financial support: US Centers for Disease Control and Prevention. Supplement sponsorship is detailed in the Acknowledgments.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

The study is dedicated to Matthew Lukwiya, who died in service to his patients during the Ebola hemorrhagic fever outbreak in Gulu, and to his family.

[a] Deceased.

Reprints or correspondence: Dr. Daniel G. Bausch, Tulane School of Public Health and Tropical Medicine, Dept. of Tropical Medicine, SL-17, 1430 Tulane Ave., New Orleans, LA 70112-2699 (dbausch@tulane.edu).

The Journal of Infectious Diseases 2007;196:S142–7

0022-1899/2007/19610S2-0005$15.00
DOI: 10.1086/520545

morning and a 0.05% solution on other visibly contaminated surfaces as needed [3].

Informed consent was obtained from the patient or guardian. A convenience sample of various clinical specimens, primarily bodily fluids, was obtained from patients with laboratory-confirmed EHF (table 1). Samples of urine, stool, vomit, and sputum were taken from plastic receptacles used by the patients at their bedsides, by use of a transfer pipette. Skin surface samples, tears, and nasal blood from a patient with epistaxis were collected with a Dacron swab that was then placed into a cryovial containing sterile PBS. Saliva, breast milk, and semen (obtained from 1 convalescent patient) were obtained by providing patients with a sterile container and asking them to produce the specimen. A body louse *(Pediculus humanus corporis)* found on a patient's clothing by the treating physician was collected with a forceps. The color and absence or presence of blood was noted for each sample. All specimens were placed into sterile cryovials and stored at ambient temperature (~25°C–30°C) in the isolation ward for the rest of the day (typically ≤6 h) before being stored in liquid nitrogen at the field laboratory established for the outbreak.

Environmental specimens. Environmental surfaces in the isolation ward that were considered to have varying probabilities of EBOV contamination were selected by researchers and clinicians familiar with EHF (table 2). All environmental samples were collected over a 2-h period in the mid-afternoon, ~6 h after the last routine cleaning. A Dacron swab was rubbed over the surface in question for ~10 s and then placed immediately into a sterile cryovial containing 1 mL of PBS. An opened and closed tube and unused swab were taken as negative

Table 1. Virus culture and reverse-transcription polymerase chain reaction (RT-PCR) results from 54 clinical samples collected from 26 patients with laboratory-confirmed Ebola hemorrhagic fever.

Sample type, phase of illness	Patients, no.	Samples, no.	Day after disease onset that sample was collected, range (mean)	Virus culture positive, no. (% sample type tested)	RT-PCR positive, no./total tested (%)	Latest day positive after disease onset
Saliva						
Acute	10	12	4–14 (6)	1 (8)	8/12 (67)	8
Convalescent	4	4	12–23 (16)	0 (0)	0/4 (0)	...
Skin[a]						
Acute	7	8	4–10 (7)	0 (0)	1/8 (13)	6
Convalescent	3	3	7–15 (12)	0 (0)	0/3 (0)	...
Urine						
Acute	5	7	5–22 (14)	0 (0)	0/7 (0)	...
Convalescent	4	4	8–40 (28)	0 (0)	0/4	...
Vomit						
Acute	1	1	NA (9)	0 (0)	0/1 (0)	...
Convalescent	1	1	NA (20)	0 (0)	0/1 (0)	...
Sputum						
Acute	1	1	NA (8)	0 (0)	0/1 (0)	...
Convalescent	1	1	NA (16)	0 (0)	0/1 (0)	...
Breast milk						
Acute	1	1	NA (7)	1 (100)	1/1 (100)	7
Convalescent	1	1	NA (15)	1 (100)	1/1 (100)	15
Stool,[b] acute	4	4	4–12 (8)	0 (0)	2/4 (50)	12
Sweat,[b] acute	1	1	NA (9)	0 (0)	0/1 (0)	...
Tears,[b] acute	1	1	NA (6)	0 (0)	1/1 (100)	6
Nasal blood,[b] acute	1	1	NA (10)	0 (0)	1/1 (100)	10
Body louse,[b] acute	1	1	NA (9)	0 (0)	0/1 (0)	...
Semen,[c] convalescent	1	2	40–45 (43)	1 (50)	1/2 (50)	40
Subtotal acute	23	38	4–22 (9)	2 (5)	14 (37)	12
Subtotal convalescent	8	16	7–45 (21)	2 (13)	2 (13)	40
Total	26[d]	54	4–45 (12)	4 (7)	16 (30)	...

NOTE. Samples are classified as either acute phase (serum ELISA antigen positive and/or RT-PCR positive) or convalescent phase (previously serum ELISA antigen positive or RT-PCR positive but now reverted to negative, often with the appearance of ELISA IgG antibody). Clinical samples were classified as acute or convalescent phase on the basis of the results of the most closely matched serum sample by date, which was a mean difference of 1.2 days (range, 0–13 days) and 7.3 days (range, 0–29 days) for acute- and convalescent-phase samples, respectively. NA, not applicable.

[a] Samples were swabbed from the hand (10) or forehead (1). The sole positive sample was from a hand.
[b] No convalescent-phase samples were available for this sample type.
[c] No acute-phase sample was available for this sample type.
[d] Both acute- and convalescent-phase samples were collected from some patients.

Table 2. Virus culture and reverse-transcription polymerase chain reaction (RT-PCR) results from 33 environmental samples.

Sample	Color	Virus culture result	RT-PCR result
Outside of ward			
Changing room wall	Clear	–	–
Changing room desk	Clear	–	–
Exterior surface of door of isolation ward	Clear	–	–
Inside ward, suspected side			
Nurse's newly placed glove	Clear	–	–
Bed frame	Clear	–	–
Instrument tray for ward rounds	Clear	–	–
Inside ward, probable side			
Air (tube opened and capped, negative control 1)	Clear	–	–
Sterile swab (negative control 2)	Clear	–	–
Intravenous fluid support pole	Clear	–	–
Light switch	Clear	–	–
Floor	Clear	–	–
Handle of 0.05% bleach solution dispenser	Clear	–	–
Nurse's clean apron	Clear	–	–
Nurse's clean glove	Clear	–	–
Clean stethoscope	Clear	–	–
Stethoscope after use	Clear	–	–
Stethoscope after use and rinsing with 0.05% bleach solution	Clear	–	–
Bed frame	Clear	–	–
Bedside chair (2 different samples)	Clear	–	–
Food bowl	Clear	–	–
Spit bowl	Clear	–	–
Skin (hand) of patient attendants (3 different samples)	Clear	–	–
Clean glove of patient attendant	Clear	–	–
Corpse decontaminated with 0.5% bleach solution	Clear	–	–
Body bag decontaminated with 0.5% bleach solution (2 different samples)	Clear	–	–
Clean mattress	Clear	–	–
Intravenous tubing	Clear	–	–
Doctor's blood-stained glove (positive control 1)	Pink	–	+
Bloody intravenous insertion site (positive control 2)	Red	–	+
Total (% of all samples)	...	0 (0)	2 (7)

controls, and swabs of samples considered to be highly probable to contain EBOV (a doctor's bloody glove and a bloody intravenous site) were collected as positive controls. The cryovials were immediately transported to the field laboratory and stored in liquid nitrogen.

Laboratory testing. A field laboratory for the diagnosis of EHF was set up at the beginning of the outbreak at St. Mary's Hospital Lacor in Gulu. Serum samples were tested by ELISA for EBOV-specific antigen and IgG antibody, as described elsewhere [9, 10]. IgM antibody testing was not possible in the field because of technical difficulties. Most serum samples were also tested at the field laboratory by conventional reverse-transcription polymerase chain reaction (RT-PCR) [11]. A patient with a laboratory-confirmed case of EHF was considered to be any person who was ELISA antigen positive or RT-PCR positive. Patients considered to be convalescent were those who previously had confirmed cases but whose ELISA antigen and RT-PCR results had reverted to negative. Most convalescent patients were ELISA IgG antibody positive.

The bodily fluids and environmental specimens collected for this study were stored in liquid nitrogen containers at the field laboratory. However, because of limited space, the specimens were periodically transported to the Uganda Virus Research Institute for temporary storage in mechanical freezers at −80°C. At the end of the outbreak, all samples were transported on dry ice in International Airline Transport Association–compliant safety shippers to the biosafety level 4 laboratory at the Centers for Disease Control and Prevention in Atlanta, Georgia, where they were catalogued and stored in liquid nitrogen until testing.

Clinical and environmental samples were tested in duplicate for virus by culture [9] and by real-time RT-PCR [11], as described elsewhere. For virus culture, 100 μL of each specimen was inoculated onto Vero E6 cell monolayers. Stool samples were first passed through a 0.22-μm filter to remove bacteria. The body louse was homogenized in a small amount of sterile PBS.

Data collection and analysis. Data were analyzed using SPSS (version 12.0; SPSS). Fisher's exact test was used when appropriate.

RESULTS

Clinical specimens. Fifty-four specimens from 26 patients, 12 (46%) of whom died, were collected (table 1). Sixteen clinical specimens from 12 patients were positive by virus culture (4 specimens) and/or RT-PCR (16 specimens), including saliva (8 of 16), skin swab (1 of 11), stool (2 of 4), semen (1 of 2), breast milk (2 of 2), tears (1 of 1), and nasal blood (1 of 1). No virus was found in urine (0 of 11), vomit (0 of 2), sputum (0 of 2), sweat (0 of 1), or the body louse (0 of 1). Three of the 16 positive specimens (2 saliva and 1 nasal blood) visibly contained blood.

As indicated by RT-PCR and ELISA antigen results from blood (data not shown), the shedding of EBOV in saliva corresponded almost exactly to the period of viremia, with the last positive saliva specimen noted at day 8 after disease onset. In contrast, specimens of breast milk and semen were found to be culture positive and RT-PCR positive at days 15 and 40 after disease onset, respectively, when EBOV was already cleared from the blood. The same patient's semen was negative when retested at day 45. Despite the fact that 7 of the 11 skin swabs were collected from patients during a period of high antigenemia (reciprocal antigen titer, ≥256) and/or RT-PCR positivity in the blood, only 1 was RT-PCR positive. All 11 urine specimens were negative by both culture and RT-PCR, even though 2 of the specimens were collected during periods of high antigenemia. Overall, mortality was not significantly different for patients with positive versus negative clinical samples, although the small sample size for most sample types calls for caution in interpretation of this finding. Mortality was significantly higher among patients with RT-PCR–positive saliva than among those who were RT-PCR negative (4 of 6 versus 0 of 7, respectively; $P = .02$).

Environmental specimens. Thirty-three environmental specimens were collected (table 2). None were culture positive, but 2 specimens (1 bloody glove and 1 bloody intravenous insertion site sampled as positive controls) were positive by RT-PCR. Both specimens were visibly colored by blood (i.e., red or pink), whereas all 31 of the negative samples were clear.

DISCUSSION

We found EBOV to be shed in a wide variety of bodily fluids during the acute phase of illness, including saliva, breast milk, stool, and tears. In most cases, the infected bodily fluid was not visibly contaminated by blood. Of particular concern is the frequent presence of EBOV in saliva early during the course of disease, where it could be transmitted to others through intimate contact and from sharing food, especially given the custom, in many parts of Africa, of eating with the hands from a common plate. However, the isolation of EBOV from only 1 saliva specimen, in contrast to the 8 that were RT-PCR positive, could suggest that the virus is rapidly inactivated by salivary enzymes or other factors in the oral cavity that are unfavorable to virus persistence and replication. EBOV has been previously documented in saliva by RT-PCR, but no attempt was made to culture virus or to explore the temporal dynamics of virus shedding in that study [12]. Marburg virus, the other member of the *Filoviridae* family, has been isolated as well as detected by RT-PCR in saliva from a patient with a fatal case of Marburg hemorrhagic fever in the Democratic Republic of the Congo (authors' unpublished data). The higher mortality among patients with RT-PCR–positive saliva likely reflects increased virus shedding in patients with high viremia, which has been previously noted to be an indicator of a poor prognosis [9, 11].

The finding of EBOV in breast milk raises the possibility of direct mother-to-child transmission. In fact, breastfed children of both of the mothers whose milk was later tested in this study died of laboratory-confirmed EHF during early stages of the outbreak. The isolation of virus from breast milk in one case even after clearance from the blood suggests that transmission may occur even during convalescence. It is possible that the mammary gland, like the gonads [5] and chambers of the eye [13, 14], is an immunologically protected site in which clearance of virus is delayed. However, we cannot rule out that the finding simply represents residual EBOV secreted into the milk during the period of viremia but not expressed until some days later, since the patient was not actively breastfeeding during admission in the isolation ward, nor can we determine whether the detected EBOV was actually a component of the milk or, rather, was contained in accompanying macrophages. At any rate, it seems prudent to advise breastfeeding mothers who survive EHF to avoid breastfeeding for at least some weeks after recovery and to provide them with alternative means of feeding their infants.

The isolation of EBOV from semen 40 days after the onset of illness underscores the risk of sexual transmission of the filoviruses during convalescence. Zaire EBOV has been detected in the semen of convalescent patients by virus isolation (82 days) and RT-PCR (91 days) after disease onset [5, 14]. Marburg virus has also been isolated from the semen and linked

conclusively to sexual transmission 13 weeks into convalescence [15].

The absence of EBOV infection in multiple tested urine specimens suggests that the virus may not be efficiently filtered in the kidney. Consequently, exposure to urine appears to be of low risk during both acute illness and convalescence. The absence of EBOV in the urine, low prevalence on the skin, and rapid clearance from the saliva in surviving patients provides some reassurance that the risk of secondary transmission from casual contacts, fomites, or the sharing of toilet facilities in the home after discharge from the hospital is minimal. This conclusion is supported by previous empirical observations [5, 6].

Abstinence from sex or the use of condoms during sex, as well as avoidance of breastfeeding and contact with the mucous membranes of the eye for at least 3 months after recovery, are still recommended to avoid possible exposure to EBOV in the aforementioned immunologically protected sites.

Other than in samples grossly contaminated with blood, EBOV was not found by any method on environmental surfaces and by RT-PCR on the skin of only 1 patient. These results suggest that environmental contamination and fomites are not frequent modes of transmission, at least in an isolation ward. However, the infectious dose of EBOV is thought to be low, and neither cell culture nor the RT-PCR assay used for EBOV in this study have not been extensively validated for use in environmental detection. Hence, the sensitivity and specificity are unknown. It is possible that EBOV was present in the environment below the threshold of detection or that environmental surfaces in the isolation ward were, at times, initially contaminated by EBOV but then decontaminated through the daily cleaning routine. However, many of the inanimate objects tested, such as bed frames and bedside chairs, would not routinely be specifically decontaminated with bleach solutions under existing guidelines unless they happened to be visibly contaminated [3], suggesting that environmental contamination did not occur. Taken together with empirical epidemiological observations during outbreaks, our results suggest that current recommendations for the decontamination of filoviruses in isolation wards [3] are effective. The risk from environmental contamination and fomites might vary in the household or other settings where decontamination would be less frequent and thorough, especially if linens or other household materials were to become visibly soiled by blood.

There was a significant discrepancy between the results of virus culture and RT-PCR testing in our study, with many more frequent positive results from RT-PCR. Possible explanations for this finding include virus degradation from breaks in the cold chain during sample collection, storage, and shipping; the greater sensitivity of RT-PCR relative to culture; and, in the case of the saliva specimens, possible virus inactivation by salivary enzymes. The less-than-ideal storage conditions of the specimens in the isolation ward immediately after acquisition and the fact that even the nasal blood from 1 patient was culture negative suggest that some virus degradation indeed occurred. Nevertheless, we cannot exclude the possibility of a true absence of viable virus in the original samples. We hope to be able to repeat this study in the future with better maintenance of the cold chain to resolve this question.

Taken together, our results support the conventional assumptions and field observations that most EBOV transmission comes from direct contact with blood or bodily fluids of an infected patient during the acute phase of illness. The risk of casual contacts with the skin, such as shaking hands, is likely to be low. Environmental contamination and fomites do not appear to pose a significant risk when currently recommended infection control guidelines for the viral hemorrhagic fevers are followed. Prospective studies with the collection of a greater number of clinical samples from patients at different stages of EHF, as well as environmental samples analyzed with an assay validated for EBOV detection in such samples, should be performed to confirm our results.

Acknowledgments

We are indebted to the patients, families, health care workers, and administrators of Gulu Regional Hospital and St. Mary's Hospital of Lacor in Gulu who made this study possible. Lastly, we thank Robert Downing at the Ugandan Viral Research Institute for his support with logistics and the cold chain.

Supplement sponsorship. This article was published as part of a supplement entitled "Filoviruses: Recent Advances and Future Challenges," sponsored by the Public Health Agency of Canada, the National Institutes of Health, the Canadian Institutes of Health Research, Cangene, CUH2A, Smith Carter, Hemisphere Engineering, Crucell, and the International Centre for Infectious Diseases.

References

1. Feldmann H, Jones S, Klenk HD, Schnittler HJ. Ebola virus: from discovery to vaccine. Nat Rev Immunol 2003; 3:677–85.
2. Leroy EM, Kumulungui B, Pourrut X, et al. Fruit bats as reservoirs of Ebola virus. Nature 2005; 438:575–6.
3. Centers for Disease Control and Prevention (CDC), World Health Organization. Infection control for viral haemorrhagic fevers in the African health care setting. Atlanta: CDC, 1998.
4. Khan AS, Tshioko FK, Heymann DL, et al. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. Commission de Lutte contre les Epidemies à Kikwit. J Infect Dis 1999; 179(Suppl 1):S76–86.
5. Rowe AK, Bertolli J, Khan AS, et al. Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. Commission de Lutte contre les Epidemies à Kikwit. J Infect Dis 1999; 179(Suppl 1):S28–35.
6. Dowell SF, Mukunu R, Ksiazek TG, Khan AS, Rollin PE, Peters CJ. Transmission of Ebola hemorrhagic fever: a study of risk factors in family members, Kikwit, Democratic Republic of the Congo, 1995.

Commission de Lutte contre les Epidemies à Kikwit. J Infect Dis 1999;179(Suppl 1):587–91.
7. Francesconi P, Yoti Z, Declich S, et al. Ebola hemorrhagic fever transmission and risk factors of contacts, Uganda. Emerg Infect Dis 2003;9:1430–7.
8. Outbreak of Ebola hemorrhagic fever Uganda, August 2000–January 2001. MMWR Morb Mortal Wkly Rep 2001;50:73–7.
9. Ksiazek TG, Rollin PE, Williams AJ, et al. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. J Infect Dis 1999;179(Suppl 1):S177–87.
10. Ksiazek TG, West CP, Rollin PE, Jahrling PB, Peters CJ. ELISA for the detection of antibodies to Ebola viruses. J Infect Dis 1999;179(Suppl 1):S192–8.
11. Towner JS, Rollin PE, Bausch DG, et al. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. J Virol 2004;78:4330–41.
12. Formenty P, Maurice Leroy E, Epelboin A, et al. Detection of Ebola virus in oral fluid specimens during outbreaks of Ebola virus hemorrhagic fever in the Republic of Congo. Clin Infect Dis 2006;42:1521–6.
13. Kibadi K, Mupapa K, Kuvula K, et al. Late ophthalmologic manifestations in survivors of the 1995 Ebola virus epidemic in Kikwit, Democratic Republic of the Congo. J Infect Dis 1999;179(Suppl 1):S13–4.
14. Rodriguez LL, De Roo A, Guimard Y, et al. Persistence and genetic stability of Ebola virus during the outbreak in Kikwit, Democratic Republic of the Congo, 1995. J Infect Dis 1999;179(Suppl 1):S170–6.
15. Martini GA, Schmidt HA. Spermatogenic transmission of the "Marburg virus" (causes of "Marburg simian disease"). Klin Wochenschr 1968;46:398–400.